Université de Sherbrooke

THE ROLE OF SURFACE LAYER PROTEINS IN *CLOSTRIDIUM DIFFICILE* BACTERIOPHAGE INFECTION

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«Mi profesión es hacer disparos al aire.
Todavía no habré descendido la primera nube.
Mas, la delicia está en curvar el arco
y en suponer la flecha donde la clava el ojo.

Yo, señor, soy acontista»

—Relato de Guillaume de Lorges, León de Greiff

RÉSUMÉ

Le rôle des protéines de surface dans l'infection des bactériophages de *Clostridium difficile.*

Maicol Ospina Bedoya. Programme de microbiologie. Mémoire présentée à la Faculté de médecine et des sciences de la santé en vue de l'obtention du diplôme de maitre ès sciences (M.Sc.) en microbiologie, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

Les phages sont des parasites bactériens présents dans tous les types d'écosystèmes et ont un effet important sur le cycle de vie des cellules procaryotes. Malgré l'importance des bactériophages dans la biologie bactérienne, leur fonction dans le cycle de vie de *Clostridium difficile* n'a pas encore été étudié de manière exhaustive. *C. difficile* est un pathogène bactérien préoccupant qui cause des infections intestinales sévères chez les humains et les animaux. Dans ce travail, nous investiguons le rôle de deux protéines de surface, CwpV et SlpA, dans l'infection de *C. difficile* par des bactériophages. La fonction de la protéine SlpA n'est pas entièrement connue. Un possible rôle dans l'infection par des bactériophages a déjà été suggéré sans toutefois avoir d'évidences expérimentales. *C. difficile* est susceptible à l'infection par des bactériophages, les récepteurs utilisés par ceux-ci sont toutefois inconnus. CwpV est la plus grosse protéine de la famille des protéines CWP chez *C. difficile*. CwpV possède une région variable en C-terminal qui est constituée de séquences répétées dont la séquence et le nombre varient selon la souche de *C. difficile* étudiée. Comme premier objectif, nous utilisons l'hôte hétérologue *Lactococcus lactis* afin de transférer l'effet de la protéine CwpV comme mécanisme de défense contre l'infection par des bactériophages. Un effet protecteur de la protéine CwpV contre le bactériophage p2 chez *L. lactis* NZ9000 (EOP = $4{,}4 \times 10^{-2}$) a été noté. De plus, des essais de survie bactérienne ont montré une réduction de la susceptibilité au bactériophage p2 des souches de *L. lactis* exprimant la protéine CwpV (environ 60%). L'expression de cette protéine n'empêche toutefois pas l'adsorption du bactériophage p2 sur la bactérie (92,8 ± 1,0 % pour le contrôle et 91,5 ± 2,9 % pour la souche test), ce qui suggère que CwpV n'interfère pas lors de l'interaction entre le récepteur primaire et le bactériophage. Dans la deuxième partie de cette étude, nous montrons par l'utilisation d'un mutant *slpA*$^-$ issus de la souche épidémique R20291 que l'absence de la protéine SlpA à la surface de la cellule rend la bactérie insensible à l'infection par trois bactériophages appartenant à la famille des *Siphoviridae*: ϕCD38-2, ϕCD111 et ϕCD146. La complémentation du mutant *slpA*$^-$ avec l'allèle de type sauvage rétabli la susceptibilité à l'infections par ces bactériophages. La réintroduction de cinq allèles provenant de différentes souches de *C. difficile* chez le mutant *slpA*$^-$ confère aussi une susceptibilité à d'autres bactériophages de la famille des *Myoviridae* qui n'infectent pas normalement la souche R20291. Finalement, la co-expression de deux allèles de la protéine SlpA dans la souche sauvage R20291 (types 4 et 12) confère une double sensibilité aux bactériophages des deux différentes familles. L'objectif de recherche de ce travail est d'approfondir et de comprendre à plus large échelle la relation hôte-bactériophage du point de vue des protéines de surface de *C. difficile*.

Mots clés: *Clostridium difficile*, *Lactococcus lactis*, bactériophage, CwpV, système d'antiphage, SlpA, récepteur

SUMMARY

The role of surface layer proteins in *Clostridium difficile* bacteriophage infection

Maicol Ospina Bedoya. Microbiology Program. Thesis presented at the Faculty of medicine and health sciences for the obtention of Master degree diploma (M.Sc.) in Microbiology, Faculty of medicine and health sciences, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

Phages are bacterial parasites that are present in virtually all ecosystems and have a massive effect on the life cycle of bacterial cells. Despite the importance of bacteriophages in bacterial biology, their function in the biology of *Clostridium difficile* has not been extensively studied. *C. difficile* is an important bacterial pathogen that causes severe intestinal infections in humans and animals. With this work, we seek to understand the role of two closely related surface layer proteins, CwpV and SlpA in *C. difficile* bacteriophage infection. The function of SlpA is still not completely understood. A possible role in bacteriophage infection has been suggested, although experimental evidence is lacking. *C. difficile* is prone to infection by bacteriophages, and the bacterial receptors used by these bacteriophages are unknown. CwpV is the largest protein of the *C. difficile* Cwp family. The variable region of CwpV is located toward the C-terminal end, and it is composed of a serine-glycine enriched flexible linker which is followed by repetitive sequences, whose sequence and number change depending on the *C. difficile* strain. The N-terminal domain possesses the cell-wall anchoring activity. Like SlpA, the CwpV protein undergoes maturation into two subunits that are re-associated in a non-covalent manner that forms a heterodimeric complex. Based on previous data from our lab on CwpV, the first objective of this study involved the use of the heterologous host *Lactococcus lactis* to transfer the antiphage functionality of CwpV against a new bacteriophage. We observed that the expression of CwpV conferred antiphage protection against bacteriophage p2 in *L. lactis* NZ9000 (EOP= $4.4x10^{-2}$). Additionally, bacterial survival assays showed a reduced susceptibility to p2 bacteriophage infection in *L. lactis* expressing CwpV (around 60 %). Also, the adsorption of bacteriophage p2 is not prevented in cells expressing *cwpV*, suggesting that CwpV does not block a primary bacteriophage receptor (92.8 ± 1.0 % for the control and 91.5 ± 2.9 % for the test strain). Using a *slpA*$^{-}$ mutant derived from the epidemic strain R20291, in the second part of our study, we show that the absence of SlpA from the cell surface renders the bacterium completely insensitive to infection by three related bacteriophages of the *Siphoviridae* family: ϕCD38-2, ϕCD111, and ϕCD146. Complementation of the mutant with a wild-type *slpA* allele restored susceptibility to bacteriophage infection. The reintroduction of five alleles conferred susceptibility to other bacteriophages of the *Myoviridae* family that typically do not infect the R20291 strain. Finally, co-expression of the *slpA* types 4 and12 in R20291 confers double susceptibility to bacteriophages of two different viral families. The main objective of this work was to better understand and examine on a broader level bacteriophage-host interactions through the surface layer proteins of *C. difficile*.

Keywords: *Clostridium difficile*, *Lactococcus lactis*, bacteriophage, CwpV, antiphage system, SlpA, phage receptor

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LIST OF ABBREVIATIONS

ATc	Anhydrotetracycline
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
CDAD	*Clostridium difficile* Associated Diarrhea
CRISPR	Clustered regularly interspaced short palindromic repeats
CDI	*Clostridium difficile* infection
CWB2	Cell_wall_binding_2
Cwp	Cell Wall Protein
CwpV	Cell Wall Protein Variable
dNTP	Deoxyribonucleotides
ECM	Extracellular Matrix Proteins
EDTA	Ethylenediaminetetraacetic acid
EOP	Efficiency of Plaquing
GC	Guanine- Cytosine
gDNA	genomic DNA
GM17	Glucose-M17
HMW-SLP	High Molecular Weight Surface layer Protein
ICTV	International Committee on Taxonomy of Viruses
M	Molar
MLST	Multilocus sequence typing
mM	Millimolar
MOI	Multiplicity of Infection
NCBI	National Center for Biotechnology Information
LMW-SLP	Low Molecular Weight Surface layer Protein
LTA	Lipoteichoic acid
LPS	Lipopolysaccharides

LB	Luria-Bertani
OD600nm	Optical density at 600nm of wavelength
PaLoc	Pathogenicity Locus
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PFU/mL	Plaque-Forming Units per Milliliter
pH	Potential of Hydrogen
PMC	Pseudomembranous Colitis
PSI	Polysaccharide I
RBP	Receptor Binding Proteins
R–M system	Restriction–modification systems
Sie System	Superinfection exclusion system
SDS	Sodium Dodecyl Sulfate
SLP	Surface Layer Protein
TAE buffer	Tris base, acetic acid, EDTA buffer
TEM	Transmission Electron Microscopy
TMP	Tape Measure Protein
TY	Tryptose-Yeast extract

CHAPTER I

INTRODUCTION

1.1. Brief insights into *Clostridium difficile* history

Although humans are not the sole organisms fighting against bacteria, in recent years science has been working on different flanks to find new weapons that can be used in the battle against the oldest inhabitants of the planet Earth. Obviously, regarding human health, the microorganisms that can cause diseases are the ones who receive the most attention. However, it is important to clarify that most of the microbes that abide in our body cannot cause disease. On the contrary, several can protect us against other microorganisms that can be harmful or cause an alteration in the health state of an individual. A pathogen is an infectious organism that can cause disease in a susceptible host. A particular case is depicted when some of these microorganisms only reach their quality as pathogens when its host suffers an alteration of the immune system or a modification of other protective factors that maintain it in a stable health state. This kind of organism is called opportunistic pathogen (NHI, 2007).

Clostridium difficile is an opportunistic pathogen in healthcare-associated infections that was discovered in 1935. However, it was only associated with a particular disease in humans in 1979. After that and during the next three decades, extensive clinical research completely associated *C. difficile* as the primary origin of CDAD (*Clostridium difficile* associated diarrhea) and posterior PMC (pseudomembranous colitis). Additionally, *Clostridium difficile* infection (CDI) was linked to antibiotic exposure in patients in Western countries. In the past, CDIs were never considered as a real menace to public health. Usually, epidemics were small and mortality and economic burden were never significant (Rupnik & Mastrantonio, 2016). Two prominent outbreaks in Canada and the USA in 2003 by the strain named *C. difficile* BI/ NAP1/027 delimited a new epidemic age in which bacterial infections caused by *C. difficile* would play a significant role (Rupnik & Mastrantonio, 2016).

1.1.1 Importance of *Clostridium difficile* research

The reemergence of new *C. difficile* strains brought economic problems to the health care systems, and in parallel, high virulence, escalation in incidence, mortality, and severity was progressively detected. Previous findings have evidenced *C. difficile* as one of the most important nosocomial-associated pathogens (along with *Escherichia coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa*) and equally CDI as one of the major public health concerns around the world (Cartman, Heap, Kuehne, Cockayne, & Minton, 2010).

Preoccupation about the future of *C. difficile* epidemics is rising due to new reports showing modification of the infection pattern from an opportunistic microorganism usually affecting elderly people and hospitalized patients under antibiotherapy, to a pathogen affecting patients with underlying clinical conditions, younger patients, and individuals without previous antibiotic exposure. Equally alarming, *C. difficile* infections are proliferating to the animal population, primarily animals utilized for food production (pigs being the most affected due to antibiotic overuse). Reports of contaminated vegetables, livestock and meats have worried scientist and health administration entities regarding the possibility of CDI as a food transmitted zoonosis (Rupnik & Mastrantonio, 2016).

C. difficile is a Gram-positive, bacillus-shaped, spore-forming strictly anaerobic bacterium. When the bacterial cells are under stress, they produce spores that are capable of resisting extreme conditions that metabolically active bacteria cannot tolerate. The spores can remain inactive, but once established in the human intestine, the spores can germinate and cause disease. The disease range from mild clinical effects to severe diarrhea and ultimately can degenerate into PMC (Rupnik, Wilcox, & Gerding, 2009).

A meta-analysis using PUBMED and EMBASE databases shows that around 8% of patients admitted to hospitals are carriers of toxinogenic *C. difficile* strains with almost six times higher risk of illness compared with patients that are admitted but not colonized (Zacharioudakis, Zervou, Pliakos, Ziakas, & Mylonakis, 2015). Contaminated hands, clinical instruments, and asymptomatic adult patients are not the sole reservoirs for the transmission of *C. difficile* within hospital facilities. Babies can also be colonized by bacteria with an

asymptomatic presence and act as reservoirs as well (Rousseau et al., 2012). In the last decade, it has been demonstrated that the gut microbiota (the network of microorganisms that abide in the gastrointestinal tissue) plays a major role in the physiological stability of the host, contributing to the gut health. *C. difficile* population dynamics is limited and controlled by the presence of other significant anaerobic bacteria. Recently, it has been shown that the capability of *C. difficile* to infect and colonize a host depends mostly on the inability of the normal gut population to retain *C. difficile* expansion through the intestinal cavity (Britton & Young, 2014; Buffie et al., 2014). Colonization is a vital part of the development of the disease and can happen because of the exposure to broad-spectrum antibiotics. It is well understood from various studies that older people, peripartum women, and children, immunocompromised patients and individuals with recent surgeries tend to have higher CDI susceptibility (Block, 2001; Cózar-Llistó, Ramos-Martinez, & Cobo, 2016).

Even with the upcoming of antibiotic cocktails and the development of better targeted antibiotic therapies (through the amelioration of patient diagnosis), one of the main characteristics of CDIs is the high recurrence rate (i.e., failure in the CDI contention by the first antibiotic treatment). These recurrences can result from relapses of the first infecting bacteria or reinfections from other *C. difficile* strains (Bien, Palagani, & Bozko, 2013; Rupnik et al., 2009).

1.1.2 Epidemics and evolution of *Clostridium difficile*

C. difficile epidemic behavior has changed in the last millennium. Over the history, CDI cases were underrepresented, and when they were occurring they were related to various types of strains. After the year 2000, numerous cases and clinical isolates have been evaluated (equally as the strain distribution on the continents) indicating the reemergence of specific strains belonging to the hypervirulent BI/NAP1/027 group. This lineage has been detected and monitored across Europe and North America (it has spread in several western countries). The strains belonging to this cluster are characterized by an overproduction of toxins A and B, production of a binary toxins and resistance to fluoroquinolone antibiotics. These changes led to an increase in the morbidity and mortality (McDonald et al., 2005; O'Connor, Johnson, & Gerding, 2009; Warny et al., 2005). However, in the last years, there has been a debate

regarding the mode of hypervirulence in the BI/NAP1/027 group. Other factors such as motility, antibiotic resistance, adherence, and sporulation can be added to the emergence of epidemic strains from this group (M. Merrigan et al., 2010; Stabler et al., 2009).

Across Canada, numerous studies have followed the historical development, morbidity, and mortality of CDI, where interestingly the province of Quebec takes a local relevance. Retrospective research of CDI in a period starting in 1991 and finishing in 2003 showed an increase in the incidence from 35.6 cases per 100,000 residents to 156.3 cases per 100,000 residents, respectively. With a similar pattern in the same period, the rate of complications during infection treatment increased from 7.1% to 18% (Pépin et al., 2004). A later study in hospitals mainly from Sherbrooke and Montreal metropolitan areas reported an incidence of 22.5 per 1,000 admissions and a 30-day attributable mortality rate of 6.8%. For the studied cases, a predominant strain from the group BI/NAP1/027 (82.2 percent of the cases) resistant to fluoroquinolones was found (Loo et al., 2005).

One of the latest studies performed in the country assessing the incidence, medical cost, and loss of productivity resulting from CDI, estimated that for the year 2012 there were around 37,932 cases. From there, Quebec had one of the highest numbers of bed days attributable to CDIs (5,560,668 bed days). Of those patients, around 73% were newly infected people, and 27% were recurrence cases. Sixty-one percent were cases with mild to moderate symptoms, and 54% of the patients were 75 years old or more. Quebec presents the highest estimated number of CDI of all Canadian provinces and territories with 16,562 cases (numbers far superior to equally developed provinces like Ontario, British Columbia, and Alberta) (Freeman et al., 2010; Levy et al., 2015).

Presently, the epidemic group BI/NAP1/027 is diminishing in proportion, and CDI has been caused by different strains (Poxton, 2013). This phenomenon also includes new strains from the 078 PCR-ribotype groups which both can be found in human patients and animals for meat production (Goorhuis et al., 2008; Knetsch et al., 2014).

1.1.3. Pathogenesis of *Clostridium difficile* infections

As we previously stated, resident gut microbiota alteration permits spore germination and colonization of the host intestinal cavity. This process starts with the vegetative cell of *C. difficile* that, after metabolic activation, evades the host immune system and multiplies. The production of toxins and virulence factors start finally causing clinical manifestation (Figure 1). In that order of ideas, it is important to understand the virulence factors and main structural features that are critical in colonization and other steps of the disease progression.

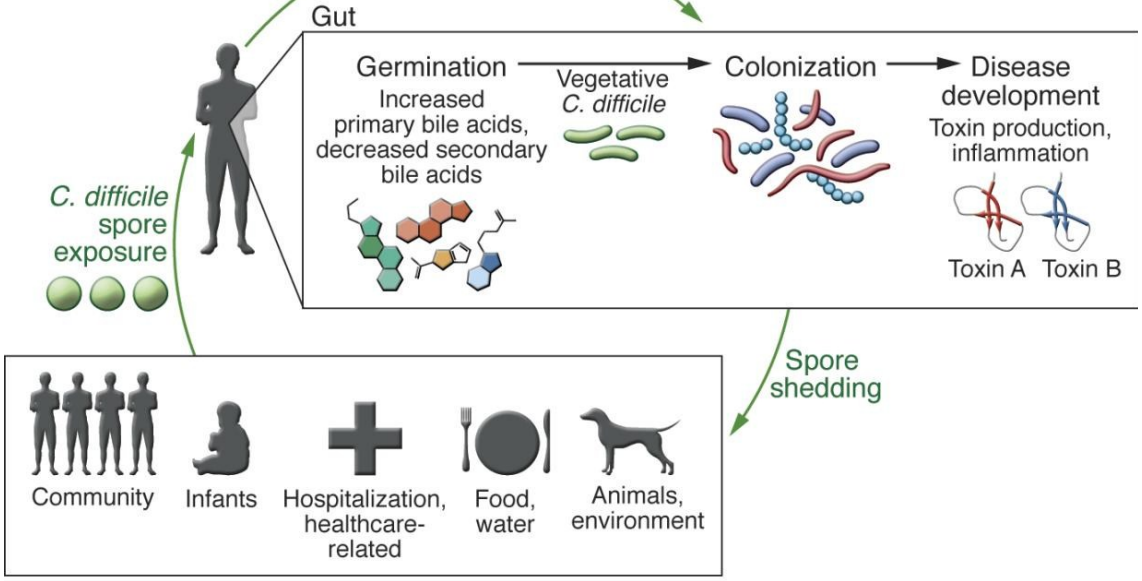


Figure 1. *C. difficile* transmission cycle. Original spore exposure from various sources does not inevitably result in disease in a healthy human. A healthy microbiota is capable of blocking *C. difficile* spore germination and vegetative growth. However, if the microbial ecology of the gut has been disturbed, spore germination and toxin production will emerge. The start of the life cycle activation can result in epithelial damage, inflammation, and apparent disease as the toxin production takes place. *C. difficile* sporulation, spore release into the environment, and transmission to new individuals continues the infectious cycle. Image taken from (Seekatz & Young, 2014).

1.2. *Clostridium difficile* virulence factors

After metabolic activation and establishment of the infection, one of the major virulence factors are produced, the exotoxins TcdA and TcdB. Both genes are located in a region of the bacterial chromosome called pathogenicity locus or PaLoc (Monot et al., 2015). The initial step of the intoxication process starts when the C-terminal receptor domain of the protein interacts with the cellular receptor (it is known that both toxins can activate a clathrin-mediated endocytosis. Also, it has been shown that the poliovirus receptor-like 3 (PVRL3) functions as a cellular factor essential for TcdB-mediated cytotoxicity). This interaction promotes the endocytosis of the toxins and posterior trafficking via endosomes. Acidification of the endosomal compartment and autocatalytic cleavage leads to the liberation of the active N-terminus into the cytosol. These domains inactivate numerous members of the Rho GTPases family which affect critical downstream cellular processes resulting in a loss of cytoskeleton integrity (Awad, Johanesen, Carter, Rose, & Lyras, 2014). Regarding the phenotypical manifestation of the cellular intoxication, the toxic effect could be compared to the outcome caused by cell apoptosis (Just et al., 1995). The PaLoc also encodes three accessory proteins named TcdR, TcdC, and TcdE. TcdE has been speculated to be involved in the release of TcdA and TcdB through permeabilization of the cell-wall. TcdR is an alternative sigma factor and TcdC may function as a negative regulator of toxin production. However, the functions of these accessory proteins remain in part controversial and are still investigated (Awad et al., 2014; Voth & Ballard, 2005).

In addition to the toxins, there are other factors like the endospore, flagella, pili, surface layer proteins, cell-wall proteins and adhesins that are equally implicated in the *C. difficile* virulence (Awad et al., 2014; Janoir, 2016b). Even if TcdA and TcdB are the primary virulence factors of *C. difficile*, other putative virulence factors may play a role in important steps of colonization, the establishment of the disease and the adherence to epithelial cells. However, these factors are also an integral part of the cellular structure and architecture of the *C. difficile* cell-wall, interaction with the environment and displacement. Some of them will be discussed in the following section.

1.3. *Clostridium difficile* cell surface architecture

1.3.1. SlpA protein

SlpA protein (for Surface-Layer Protein A) is composed of two subunits, the LMW-SLP (low-molecular-weight S-layer protein) and the HMW-SLP (high-molecular weight S-layer protein), both are a major component of the para-crystalline S-layer of *C. difficile* (E Calabi et al., 2001). The two subunits are produced from the cleavage of the protein precursor SlpA by the cysteine protease Cwp84 (described below in section 1.3.6.1) (Bruxelle et al., 2016; Dang et al., 2010). Both subunits are noncovalently re-associated after the protease action (R.P. Fagan, Albesa-Jove, Qazi, Brown K.A., & Fairweather, 2009) (Figure 2).

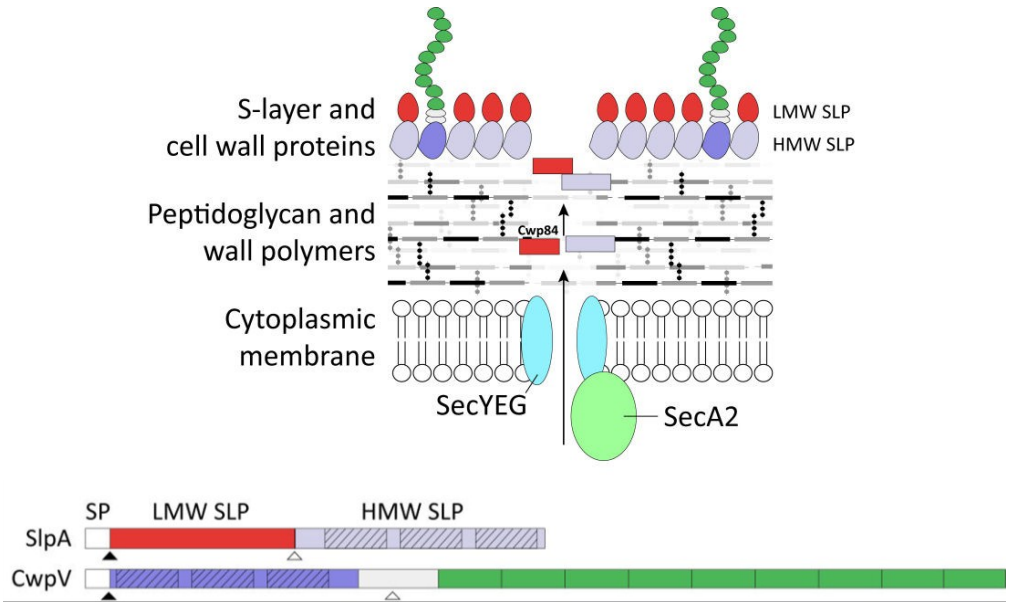


Figure 2. The *Clostridium difficile* cell wall architecture is mainly composed by a single protein, SlpA. This protein and other cell wall proteins have a dedicated secretion path (SecA2). SlpA undergoes a cleavage event, mediated by a cell wall cysteine protease Cwp84, forming the HMW and LMW SLPs which form the assembled S-layer. Image modified from (Reynolds et al., 2011a)

It has been proposed that SlpA presence has a structural role in the integrity of the bacterial shape. Different experiments have also demonstrated that both, natural SlpA proteins and recombinant SlpA, can dock to the cells from the cell line Hep-2 and also to human gastrointestinal tissues taken from healthy patients (Emanuela Calabi, Calabi, Phillips, & Fairweather, 2002). In addition, it has been proven that SlpA proteins are essential in bacterial

adherence to the colonic cell line Caco-2. Pre-incubation of bacteria with antibodies against LMW-SLP or HMW-SLP reduced adherence to Caco2 cells (M. M. Merrigan et al., 2013). Additionally, HMW-SLP presents an *in vitro* capability to attach to proteins such as type I collagen, vitronectin and thrombospondin (Emanuela Calabi et al., 2002). LMW-SLP and HMW-SLP domains can activate the host innate immune system, through the recognition of Toll-Like Receptors 4 (TLR-4). This activation stimulates signaling pathways that lead to maturation of dendritic cells and macrophage activation. However, the immunomodulatory kinetics of the SlpA proteins does not happen to correlate with the strain virulence considering that no particular immunomodulatory pattern has been discovered for the epidemic strain BI/ NAP1/027 when compared to more classical strains (Bianco et al., 2011; Poxton, 2013; Vohra & Poxton, 2012).

Studies of the phylogeny of 58 *C. difficile* isolates based on the core genome (genes common to all samples) showed the historical five clades of the population structure as determined using MLST data (K. E. Dingle et al., 2013). This pattern was also conserved in genes neighboring the *cwp* cluster (this cluster is explained in the section 1.3.6). However, in regions surrounding the genes *slpA*, *cwp66*, and *secA2*, an entirely dissimilar strain relationship appeared. A neighbor-joining phylogenetic tree of the *slpA* gene of the 58 isolates identified 12 distinct clusters (Figure 3), different from the usual five clades. Equally, neighbor-joining trees for the *cwp66* adhesin and *secA2* translocase grouped these 58 isolates identically to *slpA*. These results indicate that *slpA*, *cwp66*, and *secA2* coevolve as an independent 10-kb gene cassette, which contains at least 12 genetically divergent variants (K. E. Dingle et al., 2013). Our understanding of the role of *SlpA* in CDI (and in other fields like bacteriophage infection and virus susceptibility) has been limited by the failure to obtain a mutant, despite several attempts by different research groups. Nevertheless, using genetically modified contractile R-type bacteriocins (phage-like particles) from *C. difficile* strain CD4 to kill BI/NAP1/027-type strains (Gebhart et al., 2015), a group from AvidBiotics Corp. isolated the first mutant for the *slpA* gene in the epidemic strain R20291 (unpublished data at the time of writing these lines). This mutant has an adenine insertion early in the sequence of the gene (nucleotide position 98) that leads to a premature TAA stop codon,

creating a truncated protein (personal communication from Gregory Govoni, AvidBiotics Corp., South San Francisco, California, USA).

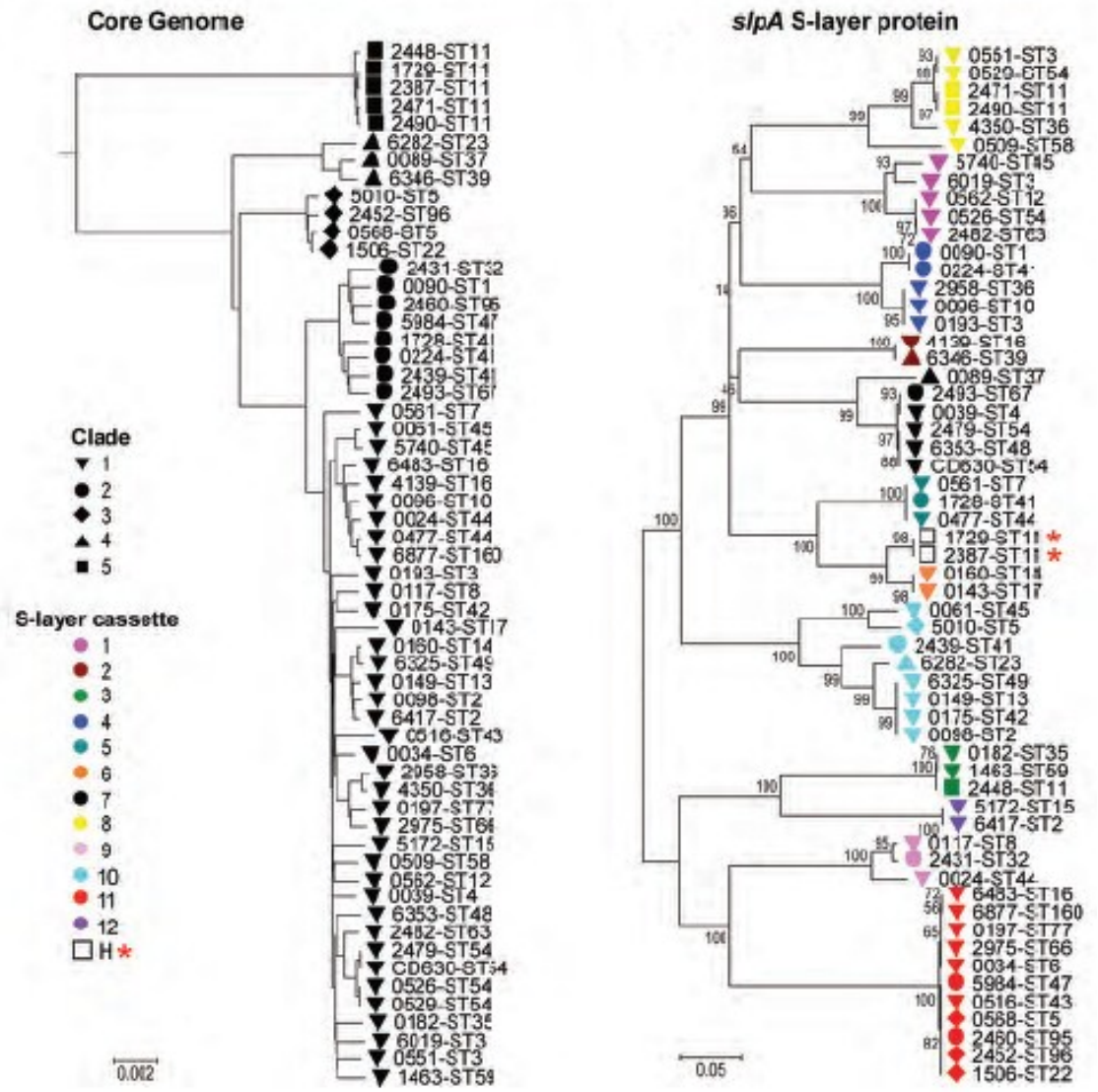


Figure 3. Phylogeny of whole genomes and *slpA*. Phylogeny created from the whole genomes: Black forms indicate the clades. Phylogeny constructed from the slpA gene: Shapes specify the clade, and colors designate the S-layer variants. Hybrids are indicated by a H and a star. Numbers indicate bootstrap support. Image taken from (Dingle et al., 2013).

1.3.2 Polysaccharides

In metabolically active *C. difficile* cells, it has been described three types of polysaccharides, PSI, PSII, and PSIII, including relevant ribotypes like 027, which has been found to contain more PSII than the other two kinds of molecules (Monteiro et al., 2013). Importantly, the presence of these molecules has stimulated the production of vaccines and technics for their use in diagnosis. Vaccines containing PSII have been tested in different animal models (Awad et al., 2014). The same molecule has been used in saccharide microarrays to detect IgG antibodies in the sera of CDI patients (Martin et al., 2013). Interestingly, new works in a mouse model and a PSII glycoprotein conjugated to TcdA and TcdB fragments indicated that antibodies targeting PSII and toxins were produced by mice, this result indicates that a combination of sugars and proteins could be a promising topic for vaccine development (Romano et al., 2014).

1.3.3. Flagella

C. difficile is motile due to the action of the flagellar machinery. A plethora of diseases-related bacteria relies on flagella as an important virulence factor (helping in tissue invasion and host colonization). Genes that regulate the assembly of the *C. difficile* flagella are ordered into three operons (Awad et al., 2014; Janoir, 2016a). The F3 locus comprises just early stage genes and contains the FliA sigma factor (also named SigD). FliA regulates the expression of late-stage flagellar genes from the F1 locus, such as genes coding for the flagellin FliC and the cap protein FliD. The F2 regulon is responsible for post-translational modification of flagella proteins that have been shown indispensable for efficient flagellar assembly and motility *of C. difficile*. The three loci show variability among lineages of *C. difficile*. Interestingly, the F3 locus is not present in the emerging virulent 078 clades, signifying in some manner that flagella and therefore motility are unessential for *C. difficile* virulence (Janoir, 2016a). One study has even shown that *fliD* mutants were more virulent than the wild-type strain (Baban et al., 2013). More data is necessary to understand the precise role of the flagellar machinery in the virulence of *C. difficile*.

1.3.4. Fimbriae and pilli

Fimbriae are not exclusive to *C. difficile* strains. In other bacteria, such as *E. coli*, *Bordetella pertussis*, *Staphylococcus* and *Streptococcus* species, the expression of fimbriae is correlated with the ability of bacteria to attach to the host and cause disease (Connell et al., 1996). However, different studies showed no correlation between serious human or animal epidemic strains, expression of fimbriae and their ability to cause disease (Borriello et al., 1988). These results suggest that at least in *C. difficile*, the expression is not necessarily prominent in the development of the disease. On the other hand, bioinformatic investigation on the phylogenetic class *Clostridia* shows that in the genome sequences of the microorganism belonging to this taxon there are several putative type IV pilus genes (Melville & Craig, 2013). Specifically, in the epidemic strains 630 and R20291 at least nine pilli or putative pilus-like genes were identified (Maldarelli, De Masi, von Rosenvinge, Carter, & Donnenberg, 2014). Recently, it has been demonstrated in historical epidemic strains 630 and R20291 that the type IV pilus contributes to biofilm formation, especially in the strain R20291. Additionally, it was also reported that this strain is capable of motility in a type IV pilus-depended manner (Purcell, McKee, Bordeleau, Burrus, & Tamayo, 2015).

1.3.5. *Clostridium difficile* adhesins

C. difficile has been shown to be able to adhere to various cell lines. For example, HT-29-MTX cells, enterocyte-like Caco-2 cells, Hep-2 cells, Vero and HeLa cells (T Karjalainen et al., 1994; Naaber, Lehto, Salminen, & Mikelsaar, 1996; O'farrely, Baird, Drudy, Fenelon, & O'Donoghue, 2001). Equally, *C. difficile* attaches to a plethora of extracellular matrix proteins (ECM) *in vitro*: fibronectin, fibrinogen, vitronectin, and type -I, -III, -IV and -V collagens (Cerquetti, Serafino, Sebastianelli, & Mastrantonio, 2002). The broad picture of *C. difficile* adhesion shows that the host-bacterium attachment requires adhesins from a bacterial origin that in general terms are proteins present on the cell surface. A plethora of adhesins has been characterized: fibronectin binding proteins, the heat shock protein GroEL, cell wall proteins (Cwp), flagella, fimbriae, etc. However, their function in the pathogenesis of CDI is still not fully understood. In the next sections, we will focus on the Cwp protein family.

1.3.6. Cwp proteins

The Cwp proteins are a group of closely related paralogous surface-associated proteins that are composed of 29 members characterized by the presence of a conserved binding domain (Pfam 04122) that attaches the proteins to the bacterial cell-wall by PSII molecules (R. P. Fagan et al., 2011; Willing et al., 2015). Almost half of the Cwp proteins are found within the "clostridial wall (*cwp*) gene cluster." This cluster has 18 open reading frames (ORFs) (Figure 4). The rest of the Cwp proteins are scattered throughout the bacterial chromosome. Different studies have shown that some of these proteins (like the protein CwpV) are secreted by the dedicated secretion system, SecA2 (K. E. Dingle et al., 2013). It is possible that other members of the Cwp family can use the same secretion system to be transported to the cell-wall. The *cwp* genes that are located inside the *cwp* cluster, like the cysteine protease Cwp84, presents high variability among *C. difficile* strains (Tuomo Karjalainen, Saumier, Barc, Delmée, & Collignon, 2002; Savariau-Lacomme, Lebarbier, Karjalainen, Collignon, & Janoir, 2003) This variability is also true for proteins like SlpA, Cwp66, and SecA2 where their coding sequences are shown to somehow be variable (regions prone to genetic exchanges by homologous recombination (K. E. Dingle et al., 2013)). In this order of ideas, the genetic variability present in Cwp surface proteins emerges from the immunological pressure from the host and contributes to its avoidance. Several of the Cwp proteins are immunogenic in patients (Drudy et al., 2004; Pechine, 2005; Pechine, Janoir, & Collignon, 2005; Wright, Drudy, Kyne, Brown, & Fairweather, 2008). Therefore, they could play an essential function in the colonization process and pathogenesis of the disease.

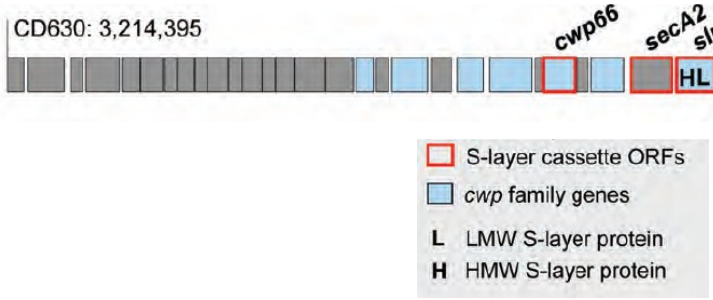


Figure 4. Genetic organization around the cwp cluster in the strain CD630. The *C. difficile* S-layer is encoded by the slpA gene, positioned within the 36.6-kb cell-wall protein (cwp) gene cluster. Image modified from (Dingle et al., 2013)

1.3.6.1. Cwp84 cysteine protease

The cysteine protease Cwp84 coding sequence is present in the *cwp* cluster and is transcribed using its promoter (Savariau-Lacomme et al., 2003). Cwp84 is found localized embedded in the bacterial S-layer and requires the processing of its pro-peptide signal by another cell-wall associated cysteine protease, Cwp13 (which is also present in the *cwp* gene cluster and can be found incorporated into the S-layer) (de la Riva, Willing, Tate, & Fairweather, 2011). Once Cwp84 is fully processed, its complete conformation is responsible for the proteolytic cleavage of the SlpA precursor. Mutants of the Cwp84 proteins present retention of proteins of the cell-wall. This mutation results in a colony phenotype in which the colony formation is not cohesive and formless (de la Riva et al., 2011). Other studies also indicate that possibly the function of Cwp84 in the disease pathogenesis is not essential due to results showing that a mutant for the protease is as virulent as the wild-type strain in a hamster model (Kirby et al., 2009).

1.3.6.2. Cwp66 adhesin

Cwp66 was the first adhesin to be identified in the class *Clostridia* (Waligora et al., 2001). The *cwp66* gene is found inside the *cwp* cluster and synthesizes a protein overproduced at the surface of the cell just after a heat-shock. It was proven to mediate adhesion to the cell line Vero (Waligora et al., 2001). As other proteins from the same cluster, Cwp66 contains two domains, one of which is highly variable. Both domains can be detected by antibodies in bacterial surface extracts. The function of Cwp66 in cell adherence was demonstrated by utilizing the same antibodies in attaching inhibition studies with the purified domains. However, no mutant for the protein has been evaluated yet and its role in the pathogenesis of the disease is unknown.

1.3.6.3. CwpV protein

Of all the members of the Cwp family, CwpV is the largest protein. Its coding sequence is not present in the *cwp* cluster. The variable region of CwpV is located toward the C-terminal end; this domain starts with a serine-glycine enriched flexible linker which is followed by

several repetitions, whose sequence and number change depending on the *C. difficile* strain (Figure 5). The N-terminal domain possesses the cell-wall anchoring activity via three CWB2 motifs that recognize PSII molecules embedded in the bacterial cell-wall (Willing et al., 2015). Like SlpA, CwpV protein undergoes maturation into two subunits that are re-associated in a non-covalent manner to form a heterodimeric complex that attaches to the bacterial cell-wall (Robert P Fagan & Fairweather, 2014; Reynolds, Emerson, de la Riva, Fagan, & Fairweather, 2011a). Interestingly, and different from SlpA, CwpV post-translational cleavage is mediated by intramolecular auto-proteolysis (this phenomenon is also present in Cwp13) (Dembek, Reynolds, & Fairweather, 2012). CwpV is exported to the cell-wall through the secA2-secretion system (Robert P Fagan & Fairweather, 2011). CwpV is a major component of *C. difficile* cell-wall, representing about 13% of the whole surface layer proteins (Reynolds et al., 2011a).

In a controlled laboratory environment, only 5% of the viable cells of the entire bacterial population express the protein. The phase variation of CwpV is regulated by DNA inversion, a mechanism of transcriptional termination by the creation of a stem-loop structure followed by a poly-U tract, which promotes transcriptional termination, in the OFF (non-transcriptional) position (Emerson et al., 2009). The C-terminal domain of CwpV presents auto-aggregative characteristics (Reynolds et al., 2011a), but its link with biofilm formation (and possible role in gut colonization) is still unknown. Future analyses are necessary to assess the biological role of CwpV. More information about the role of CwpV in bacteriophage infection can be found in the section 1.9.5.

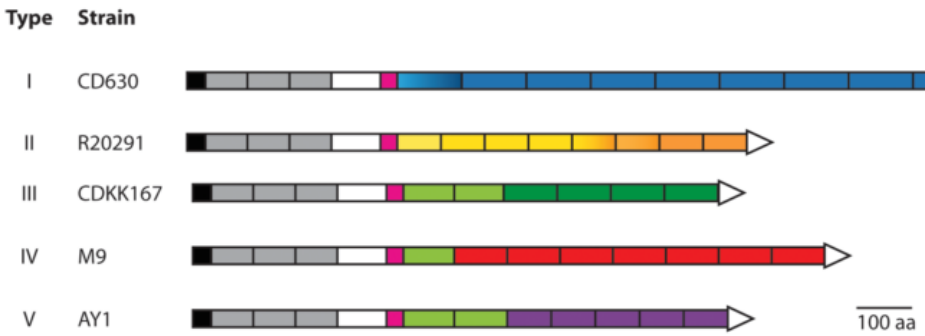


Figure 5. Visual representation of natural CwpV types. The CwpV type II from the strain R20291 was used in this study. Color code: Black, signal peptide; grey, cell-wall anchoring domain (PF04122); white, non-described function; pink, serine-glycine-rich region; blue, type I repetition; orange, type II repetition, green, type III repetition; red, type IV repetition; purple, type V repetitions. Image modified from (Sekulovic et al., 2015a).

1.4. Bacteriophages

Bacteriophages (phages) are parasites that infect bacteria and are presently viewed as the most abundant and diverse biological entities on Earth. Their vastness is not only quantitative (they surpass bacterial organisms by a ratio of 1:10), their ecology, morphology, infection strategies and other qualitative characteristics also represent relevant and exciting study fields (Brüssow & Hendrix, 2002). For example, it has been stated that only in the seas phages infect bacteria sufficiently to produce more than 10^{11} kilograms of carbon per day from natural populations, thus affecting the ecology of the planet directly (Suttle, 2007).

Phages have the capacity of killing their bacterial host. But also their genomes can transfer to the bacterial genome toxins and complementary virulence factors that can modify the host and affect human bacterial pathogens (Fortier & Sekulovic, 2013). In an identical manner, phage diversity contributes to bacterial diversity directly. Phages can function as genetic vehicles for transduction of various genes, for example, antibiotics resistance genes among different populations (Figure 6) (Grose & Casjens, 2014). In all ecosystems on the planet, a large array of phages and hosts are tangled in an endless co-evolution cycle, in which emerging phage resistant hosts continuously preserve bacterial taxa, but on the other hand,

counter-resistant phages attack these new bacterial groups. Widely speaking, phages and phage resistance mechanism have an important function in the control of bacteria in most habitats on the planet.

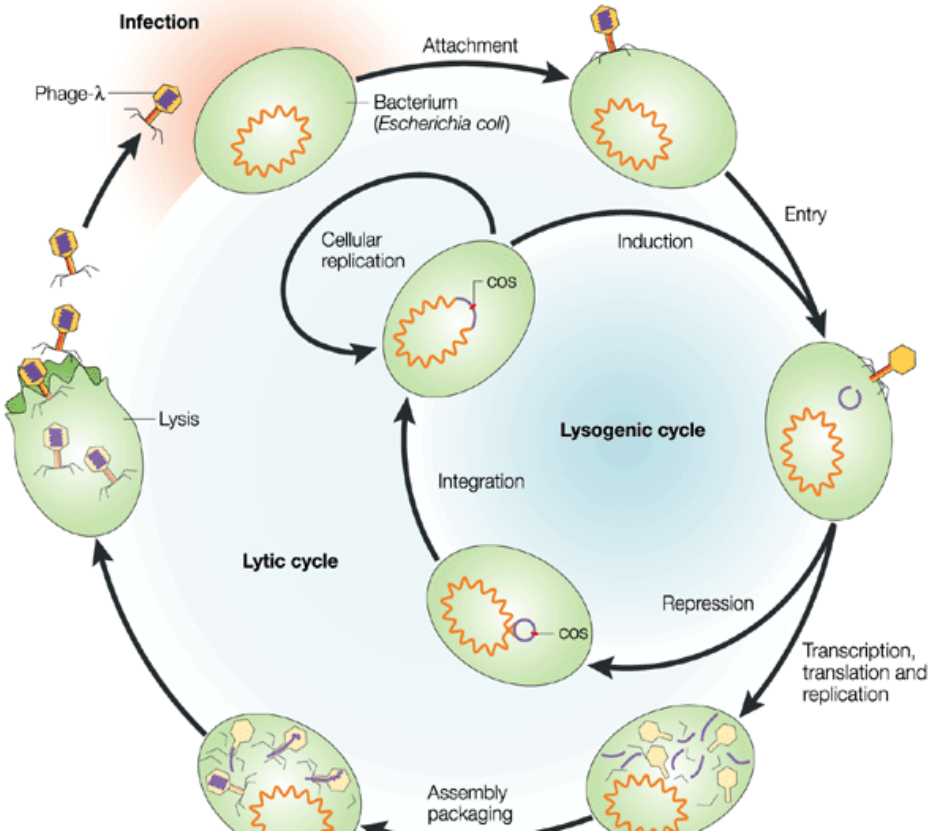


Figure 6. Illustration of the lytic and lysogenic life cycles of phage λ. Taken from (Campbell, 2003).

1.5. Bacteriophage taxonomy

At present day, bacterial viruses include at least 14 officially accepted families, and approximately 5500 phages have been isolated and investigated by electron microscopy (H.-W. Ackermann, 2007; Hans-W. Ackermann, 2009). The categorization of these phages has been essential in understanding the biodiversity and their ecological role. In the same manner, it has directed the creation of a phylogenetic tree of phages. Different from the rest of life,

viruses are polyphyletic and do not possess conserved genes among them (for example ribosomal RNA genes) that are used in phylogenetic analyses for classification purposes. In this order of ideas, phage taxonomy can be considered as a quite complicated task, also adding up the fact that phages have the capability of acquiring, transmitting, and mixing genetic material in a fast way. The International Committee on Taxonomy of Viruses (ICTV) has proposed a classification system in which it is taking into account the nature of the nucleic acids, nucleotide sequences and the viral structure of the phages (van Regenmortel, Mayo, Fauquet, & Maniloff, 2000).

From the current taxonomy, we know that most phages belong to the order *Caudovirales* and this group is divided among three main families; *Myoviridae, Podoviridae*, and *Siphoviridae*. From these families, *Podoviridae* contains around 60% of all known phages (IUMS, 2016). In a similar manner, the different phages have different morphologic features. However, they can conserve certain common structures. In fact, all phages contain their genetic material (DNA or RNA) inside a capsid that is made of protein or lipoproteins. Nevertheless, the main structural characteristic of the phage from the order *Caudovirales* is the presence of a "tail" that helps in the recognition, attachment, and injection of the DNA into the cytosolic space. It is important to remark that there are differences among the tails of the three families. The *Myoviridae* tail is relatively short but rigid and contractile, whereas the *Siphoviridae* tail is long and non-contractile. The phages of the *Podoviridae* family have a very short tail but non-contractile (H.-W. Ackermann, 2007; Hans-W. Ackermann, 2009; Grath & Sinderen, 2007; Hyman & Abedon, 2012). We show examples of the family structures in Figure 7.

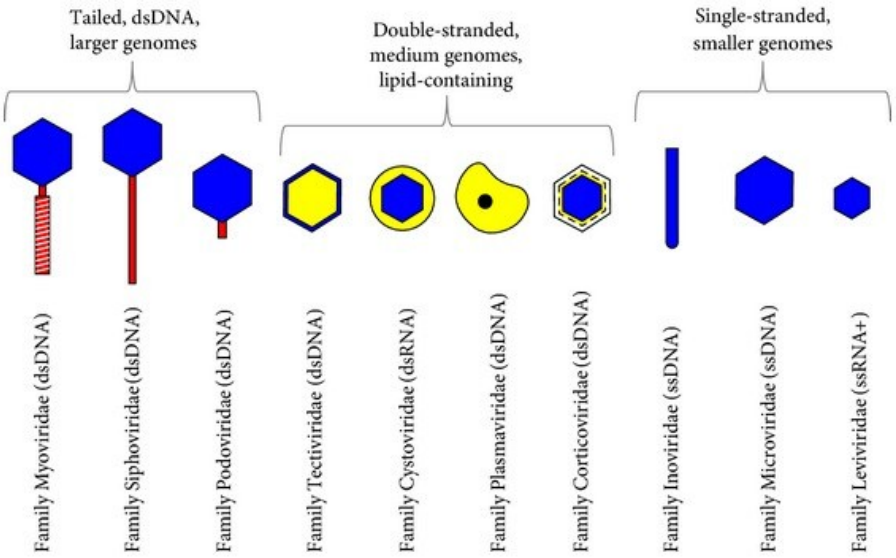


Figure 7. Diversity in bacteriophage families: Morphologies, genome natures, and comparative genome sizes. Bacteriophages are arranged in order of decreasing genome sizes. Blue coloration indicates capsids, red indicates tails, and yellow refers to lipids. Tailed bacteriophages are members of viral order *Caudovirales*. Image taken from (Hyman & Abedon, 2012).

1.6. Bacteriophage replication cycle

Despite the exceptional biodiversity that phages can bear, the mechanisms responsible for viral replication are conserved among the different families. Phages are forced to overtake the cellular machinery and metabolic processes to complete their infection cycle and achieve their replication (Grath & Sinderen, 2007).

1.6.1. Host receptors and bacteriophage adsorption

Adsorption is the first step of the infection process; the attachment of the phage to the cell surface. This initial recognition is made at first in a reversible manner by the interaction of the virion with a receptor on the surface of the bacterium. In phages with tail structures, this reversible interaction is made by the utilization of proteins localized in the distal part of the tail. Then the adsorption of the phage on the host surface becomes irreversible by the

interaction between the receptor binding proteins (RBP) of the phage and the bacterial receptors on the surface (Silva, Storms, & Sauvageau, 2016; Weinbauer, 2004). Different surface molecules are recognized to play the role of a receptor in the adsorption process, for example, the polysaccharides, lipopolysaccharides (LPS), teichoic acids, lipoteichoic acids (LTAs), and glycoproteins function as entry ports to the cell (Grath & Sinderen, 2007; Silva et al., 2016). In particular cases, phages can recognize the capsule, conjugation pilus, and flagella as means of attachment to the bacterial surface (Geoffrey William Hanlon, 2007). This natural diversity in receptors and structures of both sides reflects the evolutionary relationship between phages and bacteria.

However, just one receptor may not be involved in the attachment and adsorption; it is possible to find in nature a two-host receptor entry mechanism. Even the proteins and molecules involved in the reversible part of the adsorption could be different than the proteins and receptor involved in the irreversible part of the adsorption. For example, the phage T4 contains long tail fibers that interact with *E. coli* membrane, and this interaction is responsible for the first reversible binding. The short fibers of the virions interact with the heptose fraction of the LPS of the host and account for its irreversible binding (Silva et al., 2016). In the same manner for the Gram-positive bacterium *Bacillus subtilis*, its phage SPP1 utilizes the glycosylated cell-wall lipoteichoic acids (LTA) for a primarily reversible binding, followed by the interaction between the phage protein gp21 and the cell membrane protein YueB that determines the irreversible adsorption. It is hypothesized that the ability of a phage to engage an irreversible adsorption to molecules that are more expressed or commonly found on the cell-wall can increase the probability of finding the cellular receptor that determines its irreversible adsorption (Chatterjee & Rothenberg, 2012).

1.6.2. DNA injection

Right after the adsorption, the downstream step of phage infection is the injection of the viral DNA into the host cytoplasm. This process can be done in different ways, depending on the structural morphology of the phage that is infecting. For example, the phages from the *Myoviridae* family contract a rigid protein sheath that is capable of piercing the bacterial membrane and force the viral DNA to be released from the capsid and be translocated to the

cytosol of the bacterium. In some cases, hydrolytic enzymes can be present in the virion and degrade the cell wall polymers and facilitate viral DNA entry (González-Huici, Salas, & Hermoso, 2004; Geoffrey William Hanlon, 2007). However, although phage adsorption and DNA injection have been studied since Hershey–Chase experiments in 1952, these particular steps of the phage replication cycle are still poorly understood.

In general terms, for DNA liberation from the capsid, a signal activation must take place from the host receptor to the phage virion structure, which triggers the capsid opening and future DNA injection. Still, each phage has its unique mechanism and strategy for DNA injection. For the coliphage λ which infects *E. coli,* it is hypothesized that the viral DNA enters just by diffusion (Filali Maltouf & Labedan, 1985; González-Huici et al., 2004). Phage T4 pierces the cell membrane using its contractile tail and a cell-puncturing apparatus in the baseplate of the virions (Hans-W. Ackermann, 2009). For phage T5, the injection process has two steps: 8% of the viral DNA is translocated to the cytoplasm. From there, the proteins A1 and A2 are synthesized from the region that was transferred; these two proteins are necessary for the translocation of the rest of the viral genomes. It is thought that DNA binding domains in the proteins A1 and A2 pull the DNA into the cell (Letellier, Boulanger, de Frutos, & Jacquot, 2003). In the Gram-positive bacterium, *B. subtilis*, the phage Ø29, that has a non-contractile tail, also presents a two-step process. In the first step, DNA is injected into the cytoplasm possibly by the differential of pressure created from the virion to the cell. Thus, synthesis of early operons is activated. The second step is initiated when one of the early viral proteins, p17, participates in the molecular complex that initiates the translocation of the rest of the genome inside of the bacterium (González-Huici et al., 2004).

1.6.3. Replication cycle

In most known phages, there are two main ways of replication: the lytic cycle and the lysogenic cycle. Phages that use the lytic cycle are called virulent or strictly lytic, whereas phages that have the capability of changing their replication from the lytic to the lysogenic cycle are called temperate phages.

1.6.3.1. Lytic cycle

During the lytic cycle, phages replicate their genome producing a plethora of significant changes in the host bacterium. Ultimately, these changes cause the bacterium to stop most its cellular processes and all the cell machinery is deviated to assure the production of new viral particles. Just after the injection of the viral DNA into the cytosol of the bacterium, the bacterial RNA polymerase is recruited to transcribe early genes that are implicated in the transcription, gene regulation, DNA replication, and recombination. Afterward, late genes are transcribed, allowing the synthesis of structural proteins (capsid, tail, RBP, and so forth). In parallel, the bacterial DNA polymerase is recruited, permitting the replication of the viral genome. Altogether, viral structural proteins and DNA are assembled, forming new infective virions. The last step of the process is the liberation of the viral offspring from the intracellular space to the environment. This cellular lysis is possible by the activity of two main enzymes, the endolysin, and the holin. On one hand, the endolysins are hydrolytic enzymes that degrade the peptidoglycan cleaving different types of bonds at the interior of the polymeric structure. On the other hand, the holins are hydrophobic proteins that have the function of inserting themselves in the cytoplasmic membrane (at a precise moment of the lytic cycle) and produce pores that ultimately will allow the endolysins to reach the peptidoglycan and cleave it. After the cellular lysis, virions are released into the environment ready to find a new cell and start again the lytic cycle (Borysowski, Weber-Dabrowska, & Górski, 2006; Kutter & Sulakvelidze, 2005; Weinbauer, 2004).

1.6.3.2. Lysogenic cycle

The particularity of temperate phages is their capability to alternate between the lytic and lysogenic replication cycles. After the infection and the translocation of the viral DNA into the host cytosol, a molecular decision is taken toward one of the replication cycles. The lytic cycle was described above. On the contrary, if the phage “decides” to take the lysogenic cycle, the necessary genes for the lytic cycle are repressed, and the phage genome is integrated into the bacterial chromosome (Hans-W. Ackermann, 2009; Grath & Sinderen, 2007). Already part of the host genome, every single division of the bacterium signifies the replication of the bacterial chromosome along with the replication of the viral genome. Bacteria with an inserted phage genome are called lysogens, and the phage takes the name

of prophage. The decision regarding the infection cycle is made early after the infection, and this one is firmly affected by the condition of the intracellular environment inside the bacterium. In fact, DNA damage and unfavorable environmental conditions are known to bias the phage to take the lytic cycle (Kutter & Sulakvelidze, 2005). In that way, when a phage opts for the lysogenic cycle, some repressors are transcribed and translated to block the expression of the genes implicated in the lytic cycle (for example, CI, CII and CIII in the coliphage λ) (Anderson & Yang, 2008; Babic & Little, 2007). However, the decision of taking the lysogenic cycle is complex, and many of the mechanisms are still not understood. The enzyme integrase is also expressed and is responsible for the viral genome integration into the bacterial chromosome. Phages are remarkably stable once inserted into their host genome. However, under the right condition, it is possible to reintroduce the phage to the lytic cycle and release themselves from the host (Babic & Little, 2007; Little, 1999). Certain intracellular conditions of the host can also cause the induction of the prophage and the initiation of the lytic cycle. Stress factors like U.V light, temperature changes, the presence of different compounds (H_2O_2, antibiotics) are well known for triggering an SOS response of the lysogen. In *E. coli,* when the bacterial protein RecA is activated, its activity causes the autocleavage of the repressor of the SOS genes, LexA. The inactivation of this repressor permits then the transcription and future translation of the SOS genes, those implicated in DNA damage reparation. Equally, activation of the RecA protein leads to the proteolytic cleavage of the CI repressor, that maintains the phage in his prophage state and regulates another repressor of the lysogenic cycle. The relaxation of the expression of the repressors CI, CII, and CIII, promotes the augmentation of the expression of the protein Cro, which can activate the transcription of some genes of the lytic cycle and repress at the same time the genes from the lysogenic cycle (Court, Oppenheim, & Adhya, 2007).

1.7. Bacteriophages of *Clostridium difficile*

To date, all members of the sequenced *C. difficile* phages that have been described in the scientific literature are temperate phages of the *Caudovirales* order (phages with tail) and are classified into the *Myoviridae* or *Siphoviridae* families (where the majority of the diversity is found in the *Myoviridae* family and the *Siphoviridae* family having just a few members) (Hargreaves & Clokie, 2014, 2015; Sekulovic, Garneau, Néron, & Fortier, 2014). The

myoviruses can be sorted into three discrete morphological clades based on the capsid diameter and tail lengths. Small *Myoviridae* phages with capsid diameters of 40–60 nm and tail lengths of 105–110 nm. Intermediate sized with capsid diameters between ~60–70 nm and tail lengths of 110–130 nm. And finally, long-tailed with capsid diameter between ~60 to ~70 nm and tail lengths between 150–260 nm. All known *C. difficile* phages follow a lysogenic life cycle, their genome is characterized by the presence of predicted integrase genes, and their genome sizes range from ~31 to ~131 kbps with a GC content of 28.4% to 30.8%. The *C. difficile* phages utilized in this study are presented in Table 1.

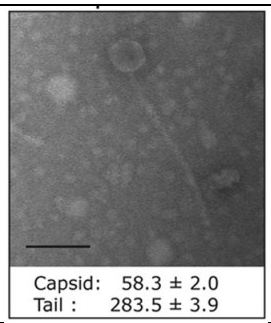
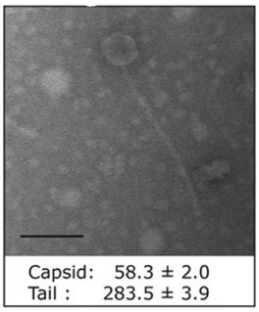
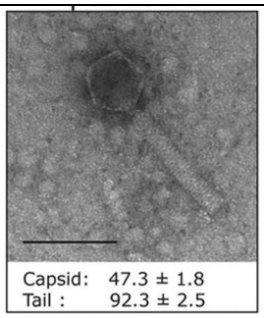
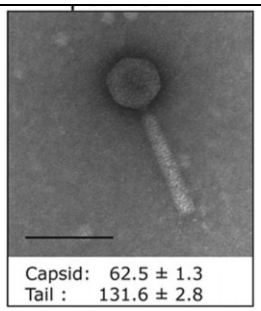
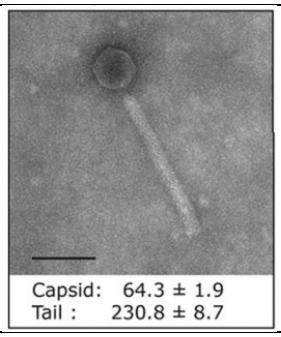
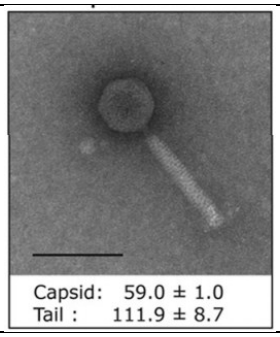
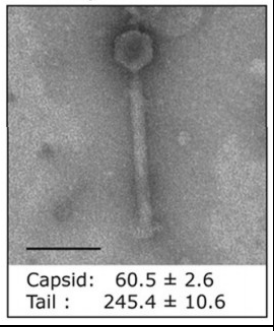
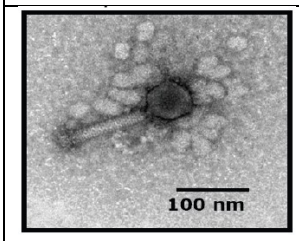
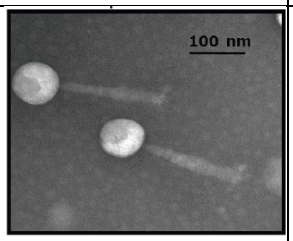
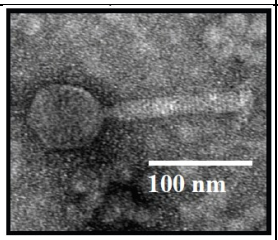
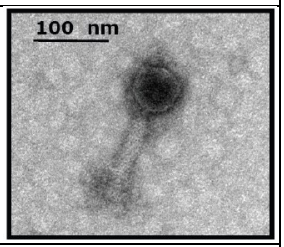
1.8. *Lactococcus lactis* and its bacteriophages

Lactococcus lactis is a microorganism that has been tamed and utilized for millennia in dairy fermentations. *L. lactis* abides in niches close to animal and plant surfaces, and is also found in the gastric cavities. However, *L. lactis* industrial strains used in cheese buttermilk, and sour cream fermentation have the ability to grow faster and rapidly produce lactic acid in milk. Although it has evident industrial interest, *L. lactis* has become an important research model among other Gram-positive bacteria for subjects such as metabolism, physiology, genetics, and molecular biology. However, at the same time, the investment in fundamental research has stimulated potential new applications such as heterologous metabolite/protein synthesis, massive culture protection, and oral vaccines (Bolotin et al., 2001).

Lactococcal phages are ubiquitous in nature where the host is present, but also in the dairy environment. The virulent nature of all lactococcal phages, their biodiversity and its adverse effect in large-scale fermentations, promoted their study around the world. Hence, several lactococcal phages have been isolated and characterized. In fact, only coliphages have been more studied than phages from the dairy industry (H. W. Ackermann, 2001). All phages from *L. lactis* known to date present a double-stranded DNA and noncontractile tail structure and fall within the *Caudovirales* order. Lactococcal phages are primarily classified in the *Siphoviridae* family and a few members are in the *Podoviridae* family. Historically, lactococcal viruses were classified using virion morphology and DNA homology index. This classification sorted dairy pages in three different main groups: the 936, c2, and P335 phages. As research techniques advanced, investigators continued with this three-group classification

but adapting multiplex PCR to rapidly assign new phages to those three groups (Deveau, Labrie, Chopin, & Moineau, 2006; S. Labrie & Moineau, 2000). The fast growth of *L. lactis* cultures permits the easy amplification and replication of phages in solid and liquid broth. The well-known secretion system of *L. lactis* also allows the intracellular expression and secretion of heterologous proteins, making this bacterium a useful model microorganism. Equally, the absence of a lysogenic cycle among its phages greatly simplifies the study of antiphage systems.

Table 1. *C. difficile* phage morphology, host, and origin of the viral particles used in this study. Virions were observed by TEM after negative staining with uranyl acetate. Phages are not displayed on the same scale. The black bars represent 100 nm. Phages Φ38-2 and ΦCD52 are not shown. When available, the Genbank accession number is indicated. NS, no-sequence. Pictures are taken from (Meessen-Pinard, 2010) and (Sekulovic et al., 2014).

ΦCD111	**ΦCD146**	**ΦCD481-1**	**ΦCD481-2**
			
Natural host: CD111 Origin: Human Family: *Siphoviridae* LN681535	Natural host: CD146 Origin: Human Family: *Siphoviridae* LN681536	Natural host:CD481 Origin: Horse Family: *Myoviridae* LN681538	Natural host:CD481 Origin: Horse Family: *Myoviridae* NS
ΦCD505	**ΦCD506**	**ΦCD508**	
			
Natural host:CD505 Origin: Dog Family: *Myoviridae* LN681539	Natural host: CD506 Origin: Dog Family: *Myoviridae* LN681540	Natural host:CD508 Origin: Dog Family: *Myoviridae* NS	
ΦMMP01	**ΦMMP02**	**ΦMMP03**	**ΦMMP04**
			
Natural host: CD19 Origin: Human Family: *Myoviridae* LN681541	Natural host: CD343 Origin: Human Family: *Myoviridae* NC_019421	Natural host:CD368 Origin: Human Family: *Myoviridae* LN681542	Natural host: CD380 Origin: Human Family: *Myoviridae* NC_019422

1.9. Antiphage systems: Infinite bacterial war

Bacteria and phages are tangled in a constant war for survival, and these interactions lead to advantageous or damaging effects in the species that are interacting. Both cannot be described and studied in isolation. As traditional ecological models show, a predatory-prey interaction (parasite-host) stimulates the continuous adaptation of the involved species to keep their fitness constant. Leigh Van Vales proposed in his "Red Queen" hypothesis that between two species, an advantageous adaptation of one species declines the fitness of the other. This fundamental evolutionary existence leads to a constant cycle of adaptation and counter-adaptation that could act as a strong force in evolution. As preys of viruses, bacteria have evolved a plethora of antiphage mechanisms to survive the constant pressure for survival. Phage resistance is a decisive phenomenon present in almost all ecological niches which can take different kinds of molecular strategies at various levels of phage infection. Below, I describe the most common antiphage systems in bacteria and how phages can overturn these mechanisms.

1.9.1. Restriction–modification systems

Restriction–modification (R–M) systems are widespread among different taxa of archaea and bacteria. Their functions are mediated by numerous heterogeneous proteins that have been sorted into at least four different groups (type I–type IV) (S. J. Labrie, Samson, & Moineau, 2010; Samson, Magadán, Sabri, & Moineau, 2013). The R-M systems are vital components of the prokaryotic arsenal against genetic parasites. When unmethylated, phage DNA enters the cytoplasm of a cell bearing an R–M system, the restriction endonuclease cleaves the foreign DNA at specific places of its sequence (restriction sites) (Vasu & Nagaraja, 2013). To evade the restriction activity on its own DNA, the host uses a methyltransferase. The host blocks the endonuclease by inserting methyl groups on the restriction sites of its DNA. Phage DNA can be also methylated. When the methylation occurs on viral DNA, the new viral particles become resistant to the cognate restriction enzyme and easily infect adjacent bacteria containing the same R–M system (Samson et al., 2013; Vasu & Nagaraja, 2013)

1.9.2. The CRISPR–Cas system

One of the most important anti-phage systems regarding biotechnological applications is the CRISPR–Cas system. The function of clustered regularly interspaced short palindromic repeats (CRISPRs) and the CRISPR-associated (*cas*) genes on phage infection and replication have been extensively described in the last decade (Boudry et al., 2015; Rath, Amlinger, Rath, & Lundgren, 2015). Phylogenetic analysis has shown that they are present in several bacterial, archaeal, and even viral genomes (S. J. Labrie et al., 2010). The locus is a composition of short repeated sequences separated by non-repetitive spacers. CRISPRs can be found in chromosomal DNA, plasmids, and phage DNA. The spacers are adapted from exogenous sequences from viruses and plasmids, giving the idea that the system could have emerged as a bacterial immunity system (Rath et al., 2015). The acquisition of news spacers renders the organism resistant to viruses containing the target sequences. When a phage-sensitive strain carrying a functional CRISPR-Cas system is infected by a virulent phage, natural phage-resistant mutants will eventually emerge. These mutants are known to have acquired at least one novel repeat-spacer unit at the 5′ end of the repeat-spacer region of a CRISPR locus (Rath et al., 2015). The newly added spacer is close or identical to a sequence called the proto-spacer which is found in the genome of the infecting virulent phage or plasmid. CRISPR activation involves a set of *cas* genes that are normally located near the CRISPR sequences. The *cas* genes encode proteins that are necessary for the recognition of invading DNA. For further details on the CRISPR–Cas system in *C. difficile* see Boudry *et al.,* 2015 (Boudry et al., 2015).

1.9.3. Blocking of bacteriophage adsorption

Adsorption to bacterial receptors is the first step of phage infection and possibly one of the most complex events, as phages must sense a particular host cellular factor. The ability to infect a host is lost when the phages cannot interact with the receptors. In normal conditions, phages encounter an enormous biodiversity in terms of the chemical composition of host membranes and cell-walls. Moreover, bacteria have developed a plethora of mechanisms to block phage adsorption. Adsorption resistance can be classified into three different

categories: the blockage of receptors, extracellular matrix production and synthesis of competitive inhibitors (S. J. Labrie et al., 2010) (Figure 8).

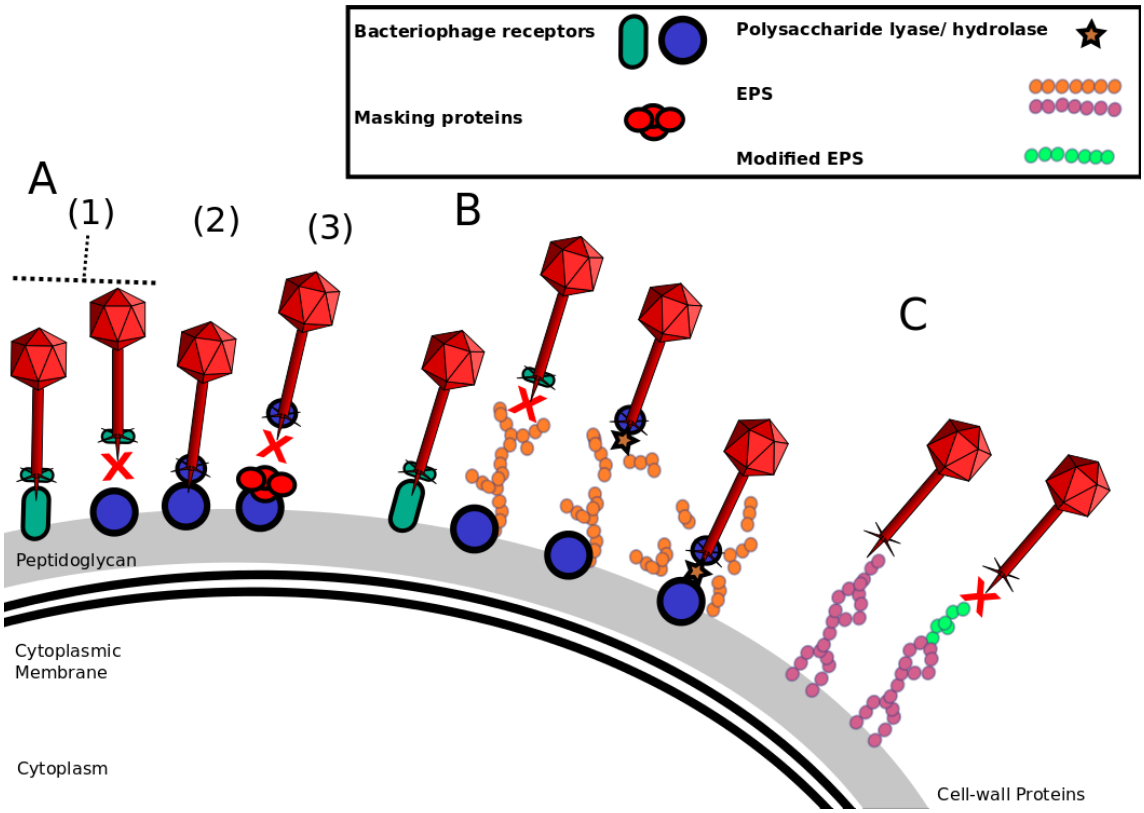


Figure 8. Strategies used by bacteria to block bacteriophage adsorption. A. Bacteriophage adsorption to the bacterial cell-wall occurs through recognition of a bacteriophage receptor on the surface. Bacteria can resist the attack by altering these surface receptors (1); bacteriophages can adapt to recognize these new receptors (2). Bacteria can also produce proteins that cover the bacteriophage receptor (3). B. Bacteriophage adsorption can be impeded by the production of exopolysaccharide (EPS), nevertheless bacteriophages can overcome the EPS coat by producing a polysaccharide lyase or a polysaccharide hydrolase to cleave the layers. C. Bacteriophages evolved to precisely identify polysaccharides. However, bacteria can modify those molecules and avoid infection.

1.9.3.1. Blockage of receptors

This first category comprises all the cases where the bacteria hide the receptors to make them not accessible to the receptor binding proteins from the phage. A representation of this could be *Staphylococcus aureus* which produces a cell-wall attached virulence factor, protein A

(Foster, 2005). It has been proven that phage adsorption is reduced when the host synthesizes the protein A. This shows that the receptor of the phage is masked by this protein (Nordström & Forsgren, 1974) (Figure 8A). A second case relates to the phage T5, that replicates in *E. coli*, and encodes a lipoprotein called Llp that can block its receptor, ferrochrome-iron receptor (FhuA). Llp is synthesized during the first part of the infection process, therefore preventing secondary infections by other phages. Equally, Llp protects newly released T5 virions from inactivation by binding to soluble receptors that are released from lysed cells. (Pedruzzi, Rosenbusch, & Locher, 1998).

1.9.3.2. Extracellular matrix production

The second category also involves masking of the receptor but through the synthesis of exopolysaccharides. The production of extracellular polymers can help in the bacterial survival in diverse environments assisting the bacteria against difficult niche conditions and even helping as a physical wall between the phages and the cellular receptor. However, some of these phages adapted to recognize these extracellular polymers (Figure 8C). Some of them even evolved the capability to degrade them (Linhardt, Galliher, & Cooney, 1986; Stummeyer et al., 2006; Ian W. Sutherland, 1999; Ian W Sutherland, Hughes, Skillman, & Tait, 2004; I W Sutherland, 1995) (Figure 8B). The enzymes used by the phages for this activity are found either linked to the structure of the viral particle (attached to the receptor binding protein) or also free enzymes released from the intracellular space when bacteria are lysed (I W Sutherland, 1995). *Pseudomonas spp*. and *Azotobacter spp*. produce alginates, that are a type of exopolysaccharide. In alginate-producing *Azotobacter spp*. bacteria, it was detected an augmentation in phage resistance (Hammad, 1998; G. W. Hanlon, Denyer, Olliff, & Ibrahim, 2001). Nevertheless, phage F116, which infects *Pseudomonas spp*., synthesizes an alginate lyase, that can assist in the diffusion of the phage through the polysaccharide matrix (G. W. Hanlon et al., 2001).

1.9.3.3. Synthesis of competitive inhibitors

The third category includes the competition that some molecules can impose to phages that attach to the same receptor (Figure 8A). In *E. coli*, the protein FhuA is an iron transporter and equally the receptor for phages such as T1, T5, and Φ80. Micromicin J25 is an antimicrobial compound that can use FhuA as an entry port to the bacterium. This molecule can compete with phage T5 for attaching to the FhuA (Destoumieux-Garzón et al., 2005). Micromicin J25 is synthesized under harsh environmental conditions and has an activity mainly in niche competition by restraining the growth of closely related strains, permitting the bacterium to escape from phage infection (Labrie et al., 2010).

1.9.4. Blocking bacteriophage DNA injection

1.9.4.1. Superinfection exclusion (Sie) systems

Superinfection exclusion is the phenomenon of a resident virus that restricts a secondary virus infection. These Sie systems are composed of molecules that interrupt the translocation of phage DNA into the bacterial cytoplasm, thus giving immunity against superinfecting phages. These molecules are predicted to be present at the cellular membrane or anchored to cell-wall components (Beperet et al., 2014). Usually, the genes that encode such proteins are present in prophages. This indication suggests that possibly the Sie systems are a way of interaction between prophages and phage from secondary infection in a single cell. Several Sie systems have been identified, but only a few of them have been characterized.

1.9.4.2. Gram-negative bacteria Sie systems

E. coli has the most characterized Sie systems, *imm* and *sp*. These two proteins can hasty block the DNA injection of the virulent phage T4 into the bacterium, avoiding future viral replication (Figure 9). Imm and Sp can act independently and have different action mechanisms. Imm prevents the translocation of phage DNA into bacterial cytosol by altering the structure of the injection port. Imm has two transmembrane domains and is predicted to be anchored to the membrane. However, Imm cannot protect by itself, and it is possibly accompanied by another membrane protein that activates its function (Lu, Stierhof, & Henning, 1993). The protein Sp inhibits the enzymatic activity of the T4 lysozyme,

preventing peptidoglycan degradation and entry of the viral DNA. The T4 lysozyme is present at the most distal part of the phage tail and pierces holes in the bacterial cell, which facilitates the injection of the phage DNA (Lu & Henning, 1994; Moak & Molineux, 2000). Also in *E. coli*, it has been proven that the phage HK97, which encodes the moron element gp15 (a moron element is an autonomously regulated DNA sequence inserted between a pair of genes that are usually adjacent in a phage genome) is responsible for the resistance to infection by other HK97 phages (independent of repressor immunity) and HK75 phages (Cumby, Edwards, Davidson, & Maxwell, 2012). Another example is the Sie system encoded by the phage φX174. This system operates in the bacterial membrane (changing a surface element that is not discovered yet) and also modifies the function of the host essential proteins for the conversion of the single stranded viral DNA to double-stranded DNA (a replicative form of the phage) (Hutchison & Sinsheimer, 1971).

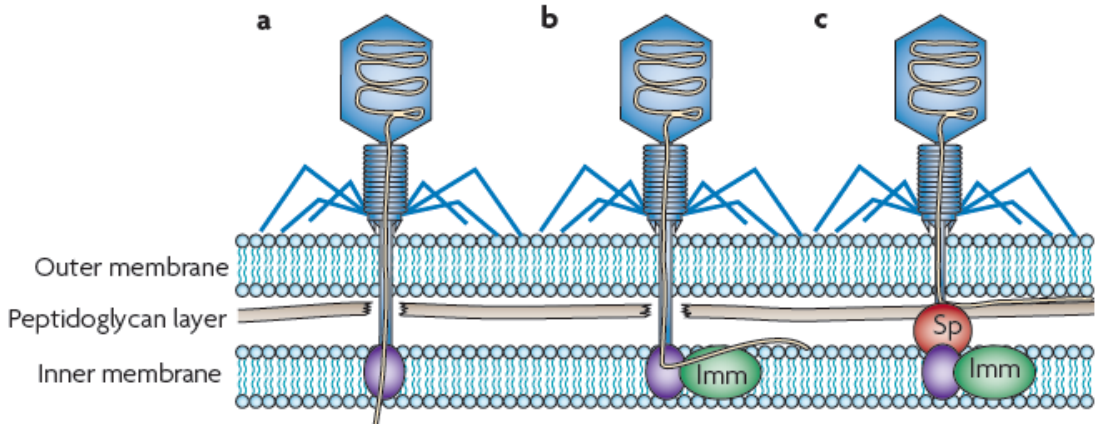


Figure 9. Blocking bacteriophage DNA translocation into the bacterial cell by a bacteriophage-encoded Superinfection exclusion (Sie) system. A. Bacteriophage T4 DNA translocation in *Escherichia coli*. The peptidogycan layer is degraded, and an inner-membrane protein is involved in the translocation of the DNA into the cytoplasm. B. Bacteriophage T4 synthetizes the protein Imm, which impedes the injection of bacteriophage DNA into the bacterial cytosol, therefore preventing infection by other T4-like bacteriophages. C. The protein Sp blocks cleavage of the peptidoglycan polymer, trapping the viral DNA between the peptidoglycan layer and the outer membrane. Image taken from (Labrie et al., 2010).

1.9.4.3 Gram-positive bacteria Sie systems

Gram-negative bacteria have been very relevant research models of study. It is necessary to fill the gap regarding other organisms and promote the research in fields where phages and Gram-positive bacteria converge. There are few examples of this kind of DNA blocking systems detected in Gram-positive bacteria. The majority was identified in *L. lactis* strains (Akçelik, 1998; Garvey, Hill, & Fitzgerald, 1996). One of the most characterized mechanism is the Sie2009, which was detected in the genome of the integrated lactococcal phage Tuc2009 and later found in other prophages integrated into the genomes of several *L. lactis* strains. The second most diverse group of lactococcal phages is the group called P335, and Sie2009 from these phages renders the bacteria resistant to an unrelated phage group called 936. Lactococcal Sie systems are predicted to be present in the cellular membrane, and they can activate phage resistance by interrupting the translocation of phage DNA into the cytoplasm (Mahony, McGrath, Fitzgerald, & van Sinderen, 2008; McGrath, Fitzgerald, & van Sinderen, 2002). Other Sie-like systems have been detected in *Streptococcus thermophilus* (Akçelik, 1998; Garvey et al., 1996). Phage TP-J34 synthesizes a 142-amino acid lipoprotein (LTP) that stops the injection of viral DNA into the bacteria. Amazingly, this mechanism gives resistance also to some lactococcal phages when transferred into the heterologous host *L. lactis* (Sun, Göhler, Heller, & Neve, 2006b). Finally, our lab has recently shown that the *C. difficile* membrane protein CwpV could protect bacteria from phage infection in a similar manner to Sie systems (further details are showed in the section 1.9.5 and 3.0)

1.9.5. CwpV protects against bacteriophage infection in *C. difficile*

In a first transcriptomic study using *C. difficile* R20291, our laboratory team isolated a lysogen containing the episomal prophage φCD38-2. In this lysogen, the *cwpV* gene was found upregulated by about 20-fold (Sekulovic & Fortier, 2015). Although it is unknown the way φCD38-2 affects CwpV synthesis, the latter observation led us to investigate *cwpV* expression.

The *cwpV* gene encodes a protein that is present in the bacterial cell wall of all *C. difficile* strains known to date (more information on CwpV is detailed in the section 1.3.6.3). The function of the protein remains unclear; however, some experimental studies suggests that

CwpV has a role in bacterial aggregation, which could ultimately impact gut colonization (Reynolds et al., 2011b). Interestingly, *cwpV* expression presents a bacterial phenomenon called phase variation. In general terms, only a part of the bacterial population synthesizes and expresses the protein (Emerson et al., 2009). The biological role of phase-variable mechanisms is not well understood, and numerous hypotheses have been proposed. Notably, one hypothesis suggests that when CwpV is expressed on the cell surface, it could interfere with phage infection (Reynolds et al., 2011b). Aligned with this hypothesis, several phage receptors, and infection resistance mechanisms are under a phase-variable regulation (Hoskisson & Smith, 2007; Seed et al., 2012). For that reason, our laboratory sought to verify if CwpV could protect the bacterial cell in a phage infection context in *C. difficile*.

Our previous studies provided experimental evidence demonstrating that CwpV protects *C. difficile* from phage infection (Sekulovic, Ospina Bedoya, Fivian-Hughes, Fairweather, & Fortier, 2015a). We showed that five types of CwpV protect against three phages from the *Siphoviridae* family; φCD38-2, φCD111, and φCD146. Similarly, the types I, III and V (types II and IV could not be tested) protects against the infection by phages of the *Myoviridae* family; φMMP01 and φCD52 (although the protection is less strong). We also showed that the antiphage activity is contained in the C-terminal part of the protein. Our data demonstrated that the viral particles are capable of attaching to the bacterium, but further phage DNA replication is not detected, strongly suggesting an inhibition at the moment of the DNA injection (Sekulovic et al., 2015a). This mechanism could be compared to the Sie systems from *L. lactis*. Interestingly, knowing that CwpV is active against two viral families can suggest that the interference involves a structure, host component or a downstream process common to both families.

The understanding of the biological and molecular mechanisms of *C. difficile* phage infection is still obscure, making the comprehension of phage-host interactions and the action of CwpV limited. Equally crucial, the bacterial receptors recognized by *C. difficile* phages were still unknown when I started this project. One of our hypothesis is that for CwpV interference to occur, the protein interacts with a structural component of the phage tail that could be

conserved among multiple phages. Alternatively, different phage families could use different injection mechanism that at one point are all affected by CwpV presence.

Objectives of the project

The general research line of this project is to examine and understand on a broader level phage-host interactions in *Clostridium difficile*, using *Lactococcus lactis* as a model microorganism to study the function of the antiphage system CwpV.

Although all previous data regarding CwpV function has been gathered using *C. difficile* as a host, this bacterial model does present several limitations. For example, the limited host range of the available phages, restrictions in the genetic manipulation of some *C. difficile* strains and lysogeny which could mask the result of phage infections. *L. lactis* represents an excellent experimental model not only because its phages are strictly lytic (and thus facilitates the study of antiphage systems), but also the receptor, structural biology and life cycle of its phages are better characterized. With our first group of objectives (#1 and #2), we seek to transfer this antiphage system to a more suitable host (heterologous host *Lactococcus lactis*) and test the antiphage properties against a well-studied phage, p2.

In a parallel manner, another limitation to our understanding of phage-host interactions is the lack of an identified host receptor used by *C. difficile* phages. In our second group of objectives (#3 and #4), we seek to understand the role of SlpA, a protein of the same family of CwpV (that shares common characteristics), in phage infection and how the different *slpA* alleles could modify sensitivity to phages. We take as guide previous studies in *C. difficile* and phage-like particles, to address a fundamental question in phage biology: finding the role of a particular protein as a determinant for phage infection.

Objective #1

Express the antiphage protein CwpV in the heterologous host *Lactococcus lactis*

Objective #2

Assess whether CwpV could confer antiphage protection against a non-phylogenetically related phage.

Objective #3

Confirm and extend our understanding of the role of SlpA as a bacteriophage receptor

Objective #4

Characterize the different *slpA* alleles in relation to sensitivity to bacteriophage infection

CHAPTER II

MATERIALS AND METHODS

2. Bacterial strains, plasmids, and bacteriophages

A comprehensive list of bacterial strains, plasmids, and phages used in this work is presented in Table 2. *L. lactis* was routinely grown in an incubator under aerobic conditions at 30°C in 0.5% Glucose-M17 (GM17) broth. Chloramphenicol (15 µg/mL) was added when necessary. *C. difficile* was grown in an anaerobic chamber (Coy Laboratories, anaerobic conditions H_2 10%, CO_2 5%, and N_2 85%) at 37°C in pre-reduced brain heart infusion (BHI) broth or TY broth (2% yeast extract, 3% tryptose, pH 7.4). Thiamphenicol (15 µg/ mL) or norfloxacin (12 µg/mL) were added when necessary. *E. coli* was grown in aerobic conditions in Luria–Bertani (LB) broth in an incubator with agitation at 37°C with proper antibiotics when necessary, chloramphenicol (25 µg/mL) or kanamycin (50 µg/mL).

Table 2. List of strains, plasmids, and phages utilized in this study.

Strain, plasmids, or bacteriophages	**Characteristic or description**	**Reference or source**
Clostridium difficile		
R20291	Epidemic isolate, ribotype 027	(Stabler *et al.* 2009)
R20291 *slpA*$^-$ mutant	R20291 mutant version of the *slpA* gene (type 4, stop codon TAA in position 98)	AvidBiotics Corp, 2015
CDMOB01	R20291 *slpA*$^-$ containing pJAK017, *slpA* type 1	This study
CDMOB02	R20291 *slpA*$^-$ containing pJAK023, *slpA* type 2	This study
CDMOB06	R20291 *slpA*$^-$ containing pJAK018, *slpA* type 6	This study
CDMOB07	R20291 *slpA*$^-$ containing pSEW027, *slpA* type 7	This study

CDMOB07b	R20291 *slpA*$^-$ containing pJAK001, *slpA* type 7b	This study
CDMOB08	R20291 *slpA*$^-$ containing pJAK019, *slpA* type 8	This study
CDMOB09	R20291 *slpA*$^-$ containing pJAK020, *slpA* type 9	This study
CDMOB10	R20291 *slpA*$^-$ containing pJAK003, *slpA* type 10	This study
CDMOB11	R20291 *slpA*$^-$ containing pAAM0013, *slpA* type 11	This study
CDMOB12	R20291 *slpA*$^-$ containing pJAK021, *slpA* type 12	This study
CDMOB13	R20291 *slpA*$^-$ containing pJAK022, *slpA* type 13	This study
CDMOBH2/6	R20291 *slpA*$^-$ containing pJAK002, *slpA* type H2/6	This study
CDMOB04Rev	R20291 *slpA*$^-$ complemented with WT *slpA* allele (type 4)	(Fagan, 2015)
Lactococcus lactis		
MG1363	Gram-positive lactic acid bacterium extensively used in dairy industry	(Gasson, 1983)
NZ9000	Derivative of MG1363 strain suitable for nisin regulated gene expression. The strain contains the regulatory genes *nisR* and *nisK* integrated into the *pepN* gene	MoBiTec GmbH 2010
L. lactis pMOB100	*L. lactis* NZ9000 containing pRPF144:*usp45:ΔSigPcwpV*	This study
L. lactis pMOB101	*L. lactis* NZ9000 containing pNZ8010:*usp45:ΔSigPcwpV*	This study
L. lactis pNZ8010	*L. lactis* NZ9000 containing pNZ8010	This study

Escherichia coli		
TOP10	Cloning strain for pRPF plasmids	Invitrogen
MC1061	Cloning strain for pNZ plasmids.	(Casadaban MJ, Cohen SN, 1980)
CA434	HB101 carrying plasmid R702 used for conjugation into *C. difficile*	(Purdy et al., 2002)
Bacteriophages		
L. lactis phage p2	*Siphoviridae*, 936 group	(Moineau, Walker, Vedamuthu, & Vandenbergh, 1995)
ΦCD38-2	*Siphoviridae*	(Sekulovic, Meessen-Pinard, & Fortier, 2011)
ΦCD111	*Siphoviridae*	(Sekulovic et al., 2014)
ΦCD146	*Siphoviridae*	(Sekulovic et al., 2014)
ΦCD481-1	*Myoviridae*	(Sekulovic et al., 2014)
ΦCD481-2	*Myoviridae*	(Sekulovic et al., 2014)
ΦCD505	*Myoviridae*	(Sekulovic et al., 2014)
ΦCD506	*Myoviridae*	(Sekulovic et al., 2014)
ΦCD508	*Myoviridae*	(Sekulovic et al., 2014)
ΦMMP01	*Myoviridae*	(Meessen-Pinard, Sekulovic, & Fortier, 2012)

ΦMMP02	*Myoviridae*	(Meessen-Pinard et al., 2012)
ΦMMP03	*Myoviridae*	(Meessen-Pinard et al., 2012)
ΦMMP04	*Myoviridae*	(Meessen-Pinard et al., 2012)
ΦCD52	*Myoviridae*	(Fortier & Moineau, 2007)
Plasmids		
pRPF144E	pRPF144 without *gusA* gene. It presents a unique BamHI site	(Sekulovic *et al.* 2015)
pRPF185E	pRPF185 without *gusA* and unique BamHI site. Expression under control of the inducible *Ptet* promoter.	(Sekulovic *et al.* 2015)
pNZ8010	pNZ8008 with a multiple cloning site (MCS1), BamHI site included	(de Ruyter *et al.* 1996)
pMOB100	pRPF144:*usp45:ΔSigPcwpV* (*cwpV* gene cloned into pRPF144E, and for which the signal peptide has been replaced by the secretion signal *usp45* from *L. lactis*)	This study
pMOB101	pNZ8010:*usp45:ΔSigPcwpV* (*cwpV* gene cloned into pNZ8010, and for which the signal peptide has been replaced by the secretion signal *usp45* from *L. lactis*)	This study
pOS200	pRPF144:*cwpV* (*cwpV* gene cloned into pRPF144E)	(Sekulovic *et al.* 2015)
pRPF233	pRPF185:*slpA* type 4	(Fagan 2015)
pJAK001	pRPF185:*slpA* type 7b	(Fagan 2015)
pJAK002	pRPF185:*slpA* type H2/6	(Fagan 2015)
pJAK003	pRPF185:*slpA* type 10	(Fagan 2015)

pJAK017	pRPF185:*slpA* type 1	(Fagan 2015)
pJAK018	pRPF185:*slpA* type 6	(Fagan 2015)
pJAK019	pRPF185:*slpA* type 8	(Fagan 2015)
pJAK020	pRPF185:*slpA* type 9	(Fagan 2015)
pJAK021	pRPF185:*slpA* type 12	(Fagan 2015)
pJAK022	pRPF185:*slpA* type 13	(Fagan 2015)
pJAK023	pRPF185:*slpA* type 2	(Fagan 2015)
pSEW027	pRPF185:*slpA* type 7	(Fagan 2015)
pAAM0013	pRPF185:s*lpA* type 11	(Fagan 2015)

2.2 Bacteriophage Amplification

Phage lysates (regularly over 10^9 PFU/mL) were prepared using standard phage amplification protocols as described previously in other studies (Cecilia Bebeacua et al., 2013; Sekulovic, Ospina Bedoya, Fivian-Hughes, Fairweather, & Fortier, 2015b). Briefly, the night before the experiment we inoculated a pre-culture of a sensitive strain in 5 mL of GM17 or BHI at 30°C or 37°C respectively. The next morning, we reinoculated a culture of 10 mL with 1% of the preculture. We followed the optical density (OD) during growth, and when the culture reached an OD of 0.1, we added salts (10 mM $MgCl_2$ and 10 mM $CaCl_2$ [only 10 mM $CaCl_2$ for phage p2]) and later the appropriate phage lysate at a multiplicity of infection (MOI) of 0.1. Again, we followed the OD looking for a decrease in the culture density, which indicates amplification of the phage. After amplification, we filtered the lysates through 0.45 μm filters and stored them at 4°C.

2.3 Bacteriophage titer determination, spot tests, and efficiency of plaquing (EOP) by Top-Agar

To assess phage titers, we used a standard soft agar overlay method (Fortier & Moineau, 2009; Sekulovic et al., 2015b). Shortly, the night before the experiment, we inoculated a preculture of a sensitive strain in 5 mL of GM17 or BHI at 30°C or 37°C respectively. In the following morning, we inoculated a culture from the pre-culture at 1% in 10 mL of fresh broth. We followed the OD at 600nm regularly. Simultaneously, we prepared GM17 or BHI

with 0.5% agar (Top-Agar) maintained at a temperature of 50°C. We serially diluted the lysates of the phages to titer. When the bacterial culture arrived at an OD_{600nm} of 0.4, we mixed in a tube 3 mL of Top-Agar, 0.5 mL of the bacterial culture, 0.1 mL of the phage lysate and salts (10 mM $MgCl_2$ and 400 mM $CaCl_2$ [10 mM $CaCl_2$ for phage p2]). We then poured the tube content over Petri dishes containing GM17 or BHI bottom agar (1% agar). We incubated overnight at the appropriate temperature, and we counted the number of phage plaques, which we multiplied by the dilution times to determine the titer of the initial lysate. For a fast evaluation of bacterial sensitivity to phage infection, we performed spot tests, in which 5 µL of serially diluted phage lysates were deposited directly over a soft agar overlay containing a suitable bacterial host. Zones of lysis in the bacterial lawn revealed a productive phage infection. The efficiency of plaquing (EOP) was used in parallel to have a quantitative analysis of bacterial sensitivity to phage infection. Briefly, the EOP is presented as the ratio of the phage titer (expressed in plaque forming units PFU/mL) of a specific phage on the test strain by the phage titer (in PFU/mL) of the same phage on a sensitive strain of reference (Sekulovic et al., 2015b). Phage titers for EOPs were calculated as mentioned above.

2.4 Cloning of CwpV in *Lactococcus lactis*

The *cwpV-II* gene was amplified by PCR from genomic DNA (gDNA) of *Clostridium difficile* R20291. The primers for this PCR and the rest of the PCR reactions are reported in Table 3. All the PCR reactions were performed using a MasterCycler thermocycler, from Eppendorf (VWR, Mississauga, ON, Canada). The PCR program, cycles, temperatures, time, and general conditions are detailed in Table 4. The PCR mix was composed of 10 ng of gDNA, 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$), 10 nM of the dNTPs mix, 1 nM of primers and 0.5 µL of PFU Turbo Polymerase. All the primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). To verify the PCR products, we analyzed them in an agarose gel 0.8% (w/v) for the fragments over 1.5kb and 2.0% (w/v) for the fragments under 1.5 kb, with TAE buffer and using Ethidium bromide staining. The fragment visualization was performed in a UV machine ImageQuant IQ 300 (GE Healthcare, Mississauga, ON). To direct the secretion and functional expression of *cwpV*, the wild type lactococcal secretion signal *usp45* from *L. lactis*, was amplified by PCR from gDNA from *L. lactis* MG1363. The products were cloned into two different backbone

plasmids, pRPF144E, and pNZ8010. The PCR products were cloned into the BamHI restriction site downstream of the promoters in both plasmids (de Ruyter, Kuipers, & de Vos, 1996; Robert P Fagan & Fairweather, 2011) (Supplementary Figure 1). In all cases, the online web tool NEBuilder (http://nebuilder.neb.com/) was used to design gene-specific primers and PCR annealing temperatures. A Gibson isothermal assembly protocol (Gibson et al., 2009) was then utilized to clone the two PCR fragments in the two plasmids backbones. The resulting plasmids were named pMOB100 and pMOB101 respectively (Table 2). Briefly, the backbone plasmids were linearized with BamHI and purified by EZ-10 spin column DNA cleanup kit (BioBasic, Mississauga, ON). Later, the plasmids were treated with 15 µL of Gibson-High Fidelity Assembly DNA cloning kit (NEB) along with equimolar ratios of *cwpV* and *usp45* fragments, giving a total volume of 20 µL. The complete reaction mix was incubated at 50°C for 1 h, and 2 µL were used to transform 50 µL of competent *E. coli* cells using standard procedures (Sambrook & Russell, 2001). In short, the reaction mix and the cells were incubated for 30 minutes on ice. After that, they were exposed to a heat-shock during 45 seconds at 42°C and put back again on ice. *E. coli* TOP10 for pMOB100 and *E. coli* MC1061 for pMOB101 were the cloning and replication hosts. Clones were tested using Colony PCR. This type of PCR was performed based on the conditions presented above but adding an additional step in which after picking a single colony from a Petri dish, this one was put directly into the PCR tube and heated for 10 minutes at 94°C. Positive colonies carrying the detected constructs were verified by DNA sequencing (CRCHUL Sequencing Platform, Québec City, Québec).

Table 3. List of primers utilized in this study

Primer number	**Target gene**	**Sequences (5' - 3')**	**Reference**	**Ta (°C)**
LCF1034	pNZ8010/*usp45* secretion signal-FWD	CAGGAGACTCTGCATGATGAAAAAAAAGATTATCTCAGCTATTTTATTG	This study	61.9
LCF1035	*cwpV-IIΔsigP/usp45* secretion signal-REV	TGTTGCCACAGTTTGAGCGTAAACACCTGACAAC	This study	

LCF1036	*usp45* secretion signal/*cwpV-IIΔsigP*-FWD	GTTTACGCTCAAACTGTGGCAACAAATTTAAC	This study	59.2
LCF1037	pNZ8010/*cwpV-IIΔsigP*-REV	AATTCTGCAGCCCGGTTATTTCATAAATCCTAGTATTCTTGATAAC	This study	
LCF1075	pRPF144E/*usp45* secretion signal-FWD	AAATAAGGAAAAAATAATAAGATGAAAAAAAAGATTATCTCAGCTATTTTAATGTCTACAGTG	This study	59
LCF1076	*cwpV-IIΔsigP/usp45* secretion signal-REV	TGTTGCCACAGTTTGAGCGTAAACACCTGACAACGG	This study	
LCF1077	*usp45* secretion signal/*cwpV-IIΔsigP*-FWD	GTTTACGCTCAAACTGTGGCAACAAATTTAACAGGG	This study	62
LCF1078	pRPF144E/*cwpV-IIΔsigP*-REV	CGGCCGTTACTAGTGTTATTTCATAAATCCTAGTATTCTTGATAACATTAATGCAATTGTAGTAG	This study	
LCF899	*cwpV-II*-FWD	NNNNGAGCTCCAGGGCAAGAAAGATATGAAACTGC	Sekulovic *et al* 2015	57.3
LCF716	*cwpV-II*-REV	GAACTCTCTCCACCTACTACATAAGATTGAG	Sekulovic *et al* 2015	
Ta, Annealing temperature				

Table 4. General PCR conditions		
Temperature	**Time**	**Cycles**
94°C	3 to 10 minutes*	Denaturation
94°C	45 sec	Denaturation
X	45 sec	Annealing
72°C	1 min/kb of DNA	Elongation
72°C	10 min	Final extension
* 10 min for the PCR performed directly on bacterial colonies that we wanted to confirm as positives for the Gibson cloning assembly. X value changes depending on the Tm of the utilized primers. 35 cycles were applied for the denaturation, annealing and elongation steps.		

2.5 Bacterial transformation

For *L. lactis*, the plasmids were transferred by electroporation (Electroporator 2510, Eppendorf) in the strain NZ9000 according to published methods (Holo & Nes, 1989). In short, the day before the experiment we inoculated *L. lactis* into 10mL of fresh GM17, and we incubated overnight at 30°C. The next day we transferred the culture into 400 mL of GM17 containing 0.5 M sucrose and 1% glycine, and we incubated at 30°C until the OD_{600nm} reached 0.2. After that, we centrifuged the cell at 8000RPM, 4°C for 10 minutes. We eliminated the supernatant and suspended the cells in 150 mL of 0.5 M sucrose + cold glycerol 10%. We centrifuged again at 8000 g, 4°C for 10 minutes (we repeated the two last steps three times to ensure the quality of the cells). We suspended the pellet in 2 mL of 0.5 M sucrose + 10% cold glycerol. Finally, we aliquoted 130 µL of the cells in tubes and frozen them at -80°C. Ready to electroporate, 40 µL competent cells were transferred into an electroporation cuvette along with 1µg of total plasmid DNA. The electroporation conditions were: 25 µF, 200Ω, and 2,5KV. Immediately after electroporation 500 µl of cold GM17 + 0.5 M sucrose, 20 mM, $MgCl_2$ + 2 mM $CaCl_2$ were added to the cells. We transferred the cells into a tube and let them rest on ice for 10 minutes. Then we incubated the electroporated cells at 30°C for 2 hours. Finally, we spread the cells on GM17 agar plates with antibiotic

and incubated at 30°C for two days. Positives colonies carrying the plasmids were verified by PCR as previously described.

2.5.1. Induction of *cwpV* expression in *L. lactis*

To induce the expression of the recombinant CwpV protein from plasmid pNZ8010 in *L. lactis*, a clone was grown in 10 mL of GM17 to an OD_{600nm} = 0.4 and induced with different subinhibitory concentrations of nisin (Sigma) at (0.1, 0.5, 1, 5 ng/mL) (de Ruyter et al., 1996; Zhou, Wang, Pan, & Li, 2008). Nisin is a 34 amino acids antimicrobial peptide that causes cell death in wild type *L. lactis*. The negative controls were wild type uninduced cells, as well as cells carrying the empty plasmid and induced with nisin. Expression of the recombinant protein at the surface of bacterial cells was confirmed by immunofluorescence as previously described (Sekulovic et al., 2015b). Briefly, bacteria from 1 mL of an overnight culture (induced and not induced) in GM17 were pelleted by centrifugation and suspended in 0.5 mL of Phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA) buffer, and later incubated for 30 minutes to block non-specific binding sites, followed by 2 hours of incubation with a rabbit anti-CwpVrptII antibody, kindly provided by Neil Fairweather (Imperial College, London, United Kingdom) (Reynolds et al., 2011a), that was diluted 1:100 in the same buffer. After two 30 second washing steps in PBS 1X, the bacteria were incubated in the dark for 45 minutes with a secondary Alexa Fluor 568-Conjugated donkey anti-rabbit IgG (H+L) antibody (Life Technologies, United States), followed by three consecutive washing steps in PBS 1X. Finally, bacterial cells were suspended in 50 µL of sterile water, spotted on glass slides and observed with an Olympus IX-81 fluorescence microscope (Olympus) equipped with a Retiga 2000R monochrome cooled CCD camera..

2.5.2. Induction of *slpA* expression in *C. difficile*

In *C. difficile* the plasmids carrying the different alleles of SlpA were transferred by conjugation as previously described (Sekulovic & Fortier, 2015) into the R20291 *slpA*$^{-}$mutant strain (Kindly provided by Gregory Govoni, AvidBiotics Corp., South San Francisco, California, USA). The plasmids contain one of the 13 SlpA types cloned from different

strains of *C. difficile*, kindly provided by Robert Fagan (University of Sheffield, Sheffield, United Kingdom) (Table 2). The cloning of the 13 types of *slpA* genes in *C. difficile* was based on the cloning vector pRPF185 containing a multicloning site under the control of the inducible P_{tet} promoter. Briefly, we started one preculture of *E. coli* CA434 and one preculture of *C. difficile* R20291 *slpA*$^{-}$. Both in 5 mL of LB (Chloramphenicol 25 + Kanamycin 50) and BHI broth respectively. The next day we took 1 mL of *E. coli* CA434, and we washed it two times with PBS 1X (centrifuging at 4000g for 5 minutes). Later we took 200 µL of the *C. difficile* R20291 *slpA*$^{-}$ culture and we mixed it with the *E. coli* pellet. We spotted the totality of the combination on BHI 1% agar Petri dishes and incubated them at 37°C overnight. The next day we recovered the bacteria with 1 mL of PBS 1X, and platted them again (100 µL) on BHI agar (thiamphenicol 15 + Norfloxacin 12) and incubated them during one night at 37°C.

The transformants were grown in 10 mL BHI to an OD_{600nm} = 0.4 and induced with anhydrotetracycline (ATc) to a final concentration of 100 ng/mL during one night. We assessed the expression of the SlpA proteins using an SDS-PAGE and performing a surface protein extraction of the induced cultures (Figure 10). The samples were prepared as follows: 10 mL of the induced *C. difficile* cultures were centrifugated at 4000 g followed by a washing step with PBS 1X. We suspended the pellet with 200 µL of glycine 0.2 M, pH = 2.2 and we incubated at room temperature for 30 minutes. We centrifugated again during 5 minutes at 10000 g. Then we transferred 150 µL of the supernatant into a new tube, and we adjusted the pH to 7.5 using a solution of Tris H-Cl 1 M, pH=7.5. We mixed 6 µL of the samples and 8µl of loading buffer 6X (Tris-HCl 10 mM pH 7.6, 0.03% Blue bromophenol, 0.7135 M (5%) β-mercaptoethanol, 60 mM EDTA and 60% glycerol). We deposited the sample in polyacrylamide gel 30% (29:1) (BioShop). The migration of the sample was performed in a Mini-Protean® Tetra Cell apparatus (Bio-rad, Mississauga, ON, Canada), using a voltage of 100 V during 1 hour and 10 minutes. The gels were visualized using a ImageQuant 300 system (GE Healthcare).

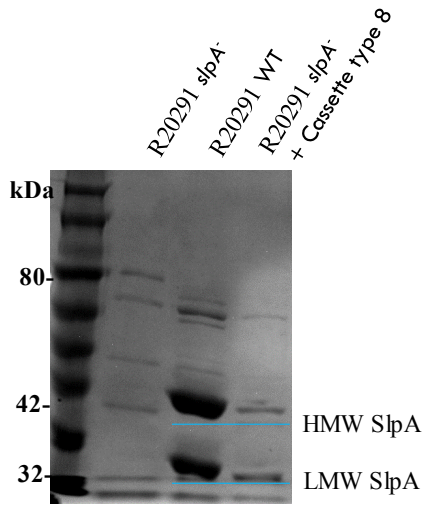


Figure 10. SDS-PAGE of SlpA extractions. The presence of SlpA in the bacterial cell wall of *Clostridium difficile* was evaluated by SDS-PAGE. In the first line, the *slpA*$^{-}$ deficient mutant where protein was not detected. In the second line, the WT strain presents the two SlpA subunits. In the third line, the cell wall extraction of the *slpA* mutant complemented with the *slpA* type 8 (CDMOB08) shows the reestablishment of the protein in the cell-wall. The same procedure was performed for all *slpA* types.

2.6 Bacterial survival assays

Bacterial survival assays were performed as previously described with slight modifications (Sekulovic et al., 2014, 2015b). In brief, *L. lactis* and *C. difficile* overnight cultures were used to inoculate 5 mL of BHI or GM17 broth and bacteria were grown close to an OD_{600nm} = 0.5, and then 0.9 ml of the culture was taken and mixed with the tested phage lysate to get an MOI of 1. $CaCl_2$ was incorporated to a final concentration of 10 mM, and finally, the volume was completed to 1 ml using fresh BHI or GM17 broth. As a standard procedure, an uninfected control without the phage was performed in parallel. In all cases, the samples were mixed by inversion and incubated for 15 minutes at 30°C for *L. lactis* and 37°C for *C. difficile*. Later the aliquots were serially diluted in triplicate. Then, 0.1 mL of the serialized dilutions were plated on GM17 or BHI agar and incubated overnight. After that, the colonies were counted, and the ratio between the infected and uninfected controls was indicative of bacterial susceptibility to phage infection since only cells that survived the infection made colonies on agar. The index was expressed as survival percentage ([infected/uninfected] × 100).

2.7 Bacteriophage adsorption assays

Phage adsorption assays were made as described previously without significant modifications (Hyman & Abedon, 2009; Sekulovic et al., 2014, 2015b). Briefly, bacteria from an overnight culture were started in GM17 or BHI broth and grown until exponential phase (OD_{600nm} = 0.5). Then, 0.9 mL of culture was mixed with $1x10^4$ PFU of the desired phage in the presence of salts ($MgCl_2$ and $CaCl_2$ for *C difficile* and or $CaCl_2$ for *L. lactis*), and the culture was completed to 1 mL with sterile BHI or GM17 broth. Phages were allowed to adsorb for 30 minutes at 37°C for *C. difficile* and 30°C *for L. lactis*. Afterward, the cells were collected by centrifugation during 2 minutes. Free phages in the supernatant that did not adsorb were enumerated on standard soft agar overlays as described earlier and titers were analyzed against the initial phage input. The adsorption percentage was calculated using the following formula: 100 – ([residual titer/ initial titer] × 100).

CHAPTER III

IMPACT OF CWPV EXPRESSION ON BACTERIOPHAGE INFECTION IN *LACTOCOCCUS LACTIS*

3. CwpV protects *Lactococcus lactis* from bacteriophage infection

With the objective of verifying if in a heterologous host the expression of CwpV could protect against phage infection, we used the *L. lactis* strain NZ9000. This strain is derivative of the MG1363 line. It is used in standard nisin-regulated gene expression and is also susceptible to infection by the lactococcal phage p2 (*Siphoviridae* phage from the 936 group) (Moineau et al., 1995). We also used the wild-type strain MG1363 to replicate the phage and have a concentrated fresh lysate.

Protein expression is one of the most important steps when testing any type of specific antiphage activity. As we wanted to ensure this part of the process, we cloned the R20291 type II *cwpV* gene (*cwpV-II*) in two different plasmids; pRPF144E and pNZ8010. The first one is a *C. difficile* plasmid under the control of the constitutive promoter P*cwp2*. The second plasmid is a *L. lactis* native plasmid under the control of a nisin inducible promoter. The presence or absence of *cwpV* expression in the strains was confirmed by immunofluorescence (Sekulovic & Fortier, 2015; Sekulovic et al., 2015b). As a pilot test, first we transformed by electroporation into *L. lactis* the plasmid pOS200 (Sekulovic, Ospina Bedoya, Fivian-Hughes, Fairweather, & Fortier, 2015a), a pRPF144E derived vector where a complete wild-type gene *cwpV-II* was cloned. The primary objective of this test was to determine if a *C. difficile* plasmid could be maintained into *L. lactis*, which would simplify translation of all experiments from *C. difficile* to *L. lactis* afterwards. However, preliminary immunofluorescence data showed no protein expression (data not shown). Taking into account previous data regarding protein expression in *L. lactis* (Borrero et al., 2011), the wild-type lactococcal secretion signal *usp45* was then cloned in the vector replacing the native signal peptide of *cwpV* with the objective of directing the secretion and functional expression of the protein. The resulting vector was named pMOB100 and transferred by

electroporation into NZ9000 that does not have the *cwpV* gene. Its constitutive expression was assessed by immunofluorescence. Expression of CwpV from the pMOB100 plasmid showed clusters of bacteria expressing the protein, nevertheless, it was clear that expression was not uniform in the whole culture and only some expression spots were detected through all the cells (data not shown). There was also aggregation of the cells in the strain expressing *cwpV*, suggesting that the absence of a clear signal in immunofluorescence could be due to a technical problem (data not shown).

Given that it is possible that some undetectable amount of protein might be present in the cellular surface, we decided to test for phage susceptibility. We could detect in a preliminary spot-test assay a protection of about 3 logs in the strain containing the plasmid pMOB100 (Figure 11), suggesting that the expression was probably sufficient to provide some protection. In summary, this experiment first confirmed that, although not optimal, the pRPF144E plasmid from *C. difficile* can be maintained in *L. lactis,* and second, the plasmid can be used to express proteins in a constitutive manner in this host.

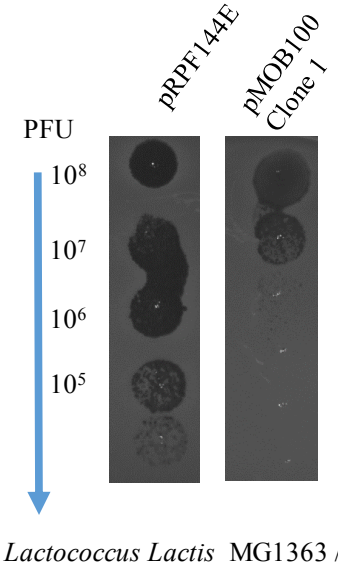


Figure 11. Susceptibility of *Lactococcus lactis* to bacteriophage infection in spot-test assays. Bacterial monolayers were prepared using cells carrying the pMOB100 plasmid expressing *cwpV-II* or the empty plasmid pRPF144E. Areas of clearing after overnight incubation shows susceptibility to bacteriophage infection. The spot-test assay was repeated three times and a representative picture is shown.

Keeping in mind the preliminary results, we wanted to improve the expression of CwpV by cloning the gene in the inducible vector pNZ8010 but maintain the cloning strategy where the *cwpV* signal peptide was replaced by the lactococcal secretion signal *usp45*. The resulting vector was named pMOB101 (Table 2) and transferred by electroporation into NZ9000. Expression of the protein was again assessed by immunofluorescence (Figure 12). Expression of CwpV from the pMOB101 plasmid showed a significant improvement and could be detected in most of the cells in the culture. It is important to underline that the protein expression was still not equal to the levels detected in *C. difficile*, and there is still room for adjustments in the expression strategy in *L. lactis* (further discussion on cellular aggregation is expanded in section 3.4). Equally important, immunofluorescence protocol for CwpV detection still could be optimized and adapted to *L. lactis* in order to reach similar results as observed with *C. difficile* (Sekulovic et al., 2015a). Also, alternative strategies as Western Blotting could be implemented for detection of CwpV expression. In summary, we could express the CwpV protein in the heterologous host *L. lactis*.

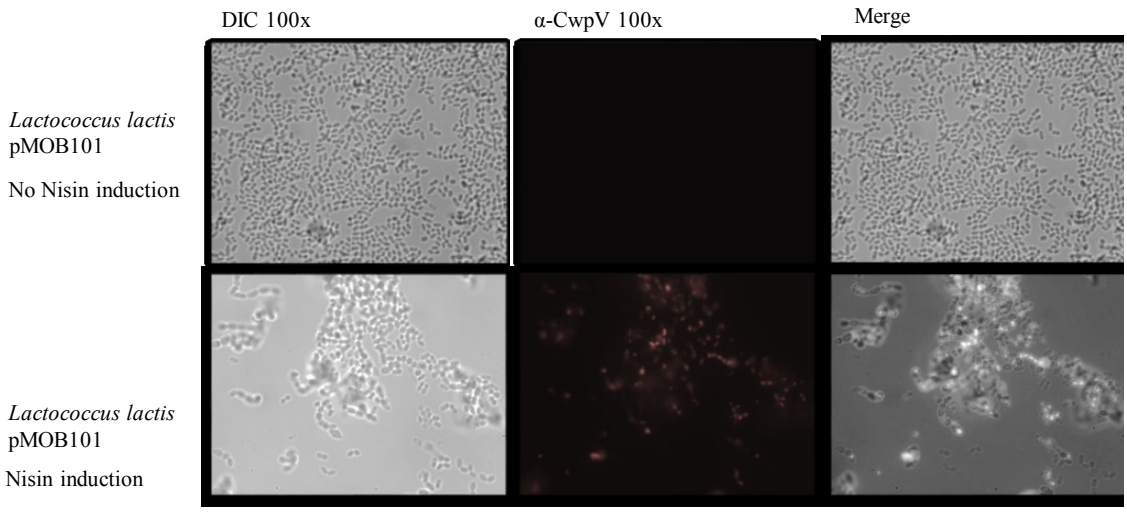


Figure 12. Immunofluorescence detection of CwpV expression. The presence of CwpV in the bacterial cell wall of *L. lactis* was evaluated by immunofluorescence using antibodies specifically directed against the C-terminal part of the protein. We can notice there is no fluorescent signal of the protein in the strain that it is not induced with nisin.

Subsequently, using a spot test infection assay, we evaluated the outcome of the expression of CwpV-II on the susceptibility to infection by the phage p2. The strain NZ9000 was completely sensitive to the infection by the phage as it was described in *C. difficile* R20291 (Sekulovic & Fortier, 2015; Sekulovic et al., 2014, 2015b). We observed a strong reduction (almost complete absence) of infection in *L. lactis* cells that were expressing *cwpV-II* (Figure 13).

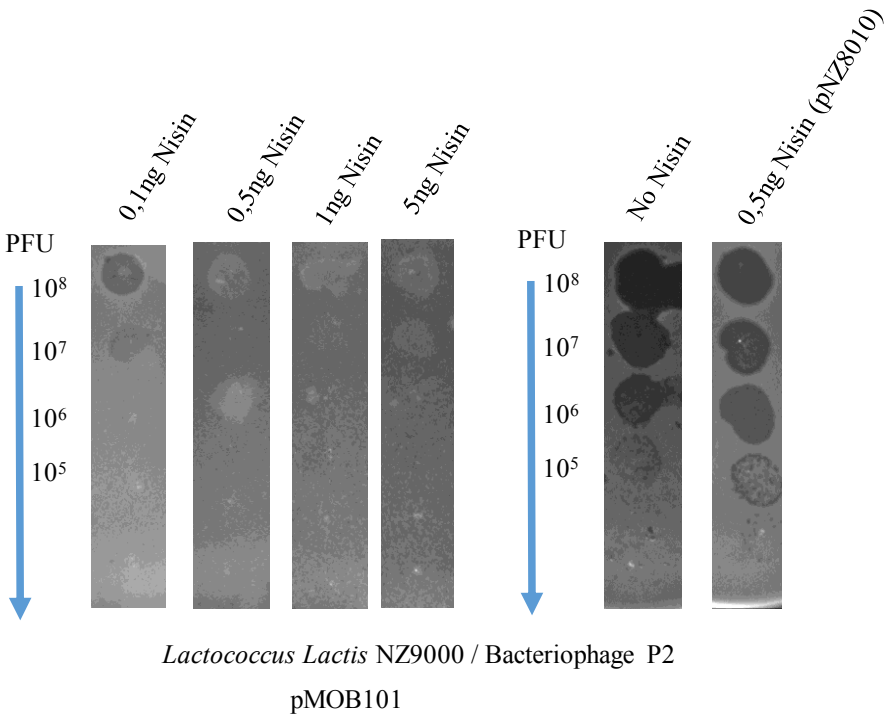


Figure 13. Susceptibility of *L. lactis* to bacteriophage infection in spot-test assays. Bacterial monolayers were prepared with the strain NZ9000 carrying the pMOB101 plasmid expressing cwpV type II or the empty plasmid pNZ8010 (also induced with 0.5 ng of nisin). Areas of clearing after overnight incubation shows sensitivity to bacteriophage infection. The spot-test assay was repeated three times and an illustrative picture is shown.

We confirmed as well that the level of resistance was proportional to the degree of protein induction. It is important to clarify that in a context of a spot-test assay, the multiplicity of infection (MOI) is particularly high (usually the lysates used in the experiments present high titers). Therefore, we decided to assess the effect of *cwpV-II* expression on the susceptibility to phage infection with parameters where the viruses and bacteria were in a ratio of 1:1 (MOI = 1). Then, we performed a bacterial survival assay in which we infected *L. lactis* (previously induced with 5 ng/mL of nisin) in broth medium with the phage p2, followed by plating of the bacteria that were infected. We then counted the colonies, representing residual viable cells. In Figure 14 we show that in *L. lactis* expressing *cwpV-II*, bacteria survived the

infection in a proportion of about 55.9 ± 12.4 %. On the other hand, only 6.3 ± 3.0 % of the bacteria bearing the empty plasmid pNZ8010 survived. In a similar manner, we infected *L. lactis* containing the plasmid pMOB101 but without induction, and in this case 21.3± 5.7 % of bacteria survived (this suggests a certain level of leakage of the inducible promoter). In this order of ideas, we confirmed that in a heterologous host the expression of *cwpV-II* confers phage protection. It is important to remark that we used an MOI of 1 in our bacterial survival test. We wanted to better access phage resistance in an infection context, and also take advantage of the nature of our phage, which is strictly lytic. This characteristic helped us to circumvent previous problems like lysogeny and resistance by previous phage infection (Sekulovic et al., 2015b).

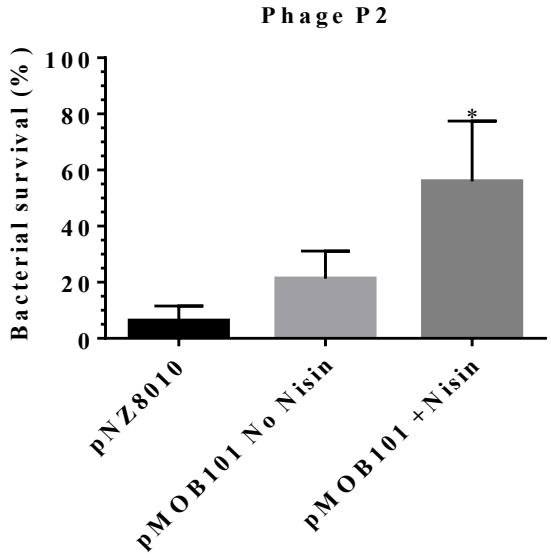


Figure 14. Bacterial survival assay following infection with lactococcal bacteriophage p2. Bacteria were mixed with bacteriophage p2 in a ratio 1:1 (MOI of 1). The strain NZ9000 carrying the plasmid pMOB101 (nisin induced and not induced) was compared with the strain NZ9000 carrying the empty plasmid pNZ8010. Colonies representing bacteria that survived the infection were counted and the index is expressed as a percentage of the ratio between infected and uninfected controls. Bars denote means ± standard deviation (SD) of three independent biological replicates, which were each plated in technical triplicates. One-way ANOVA comparisons were done with NZ9000 as the reference condition (*$P < 0.05$).

3.2 CwpV protects against infection by the siphophage p2 when expressed in *Lactococcus lactis*

To further characterize and compare the CwpV protein properties against phages, we determined the efficiency of plaquing (EOP) (Fortier & Moineau, 2009; Sekulovic et al., 2014). We incorporated into soft agar overlays dilutions of phage p2 lysates. With this, we could calculate the EOP in the *L. lactis* strain NZ9000 (in general terms an EOP is the proportion of phages that can infect a determined strain, compared to a reference strain, in this case, the wild-type host MG1363). CwpV blocked the infection against the siphophage p2, with an EOP value of 4.4×10^{-2}.

3.3 Adsorption of bacteriophage p2 is not prevented in cells expressing *cwpV*

The last experiments suggest that when CwpV is expressed, phage progeny is affected (fewer plaques). Nevertheless, phage infection could be overridden at many steps of the viral lifecycle. Taking into consideration the localization of CwpV in the bacterial cell wall, we revisited a previous hypothesis tested in our laboratory (Sekulovic et al., 2015b), which is whether CwpV could block phage adsorption. Adsorption is the first step of a viral infection where the viral particle attaches to the cell. Interference with this important step abrogates the whole infection process. To test this hypothesis, we performed a phage adsorption assay with a *L. lactis* strain expressing *cwpV-II* from the plasmid pMOB101 (previously induced with 5 ng/mL of nisin). A positive control of adsorption consisted in a strain that contained the empty plasmid pNZ8010 and does not synthesize CwpV at all. In Figure 15 we demonstrated that the adsorption levels were almost identical in the strain expressing CwpV or the one which did not, with 92.8 ± 1.0 % and 91.5 ± 2.9 % respectively. These results confirm that the expression of *cwpV* does not affect the attachment of the viral particles to the bacterial surface, thus confirming previous results (Sekulovic et al., 2015b), and that the antiphage activity involves a downstream process in the infection cycle. Also, it is known from previous research that CwpV does not interfere with phage DNA replication or early gene transcription when the phage exists in a prophage state in the bacterial genome (Sekulovic et al., 2015b). Consequently, taken together with all the data we corroborate that

CwpV might function as a non-prophage encoded superinfection exclusion system (Sie) and that this system can be transferred to a heterologous host to interfere with phage infection.

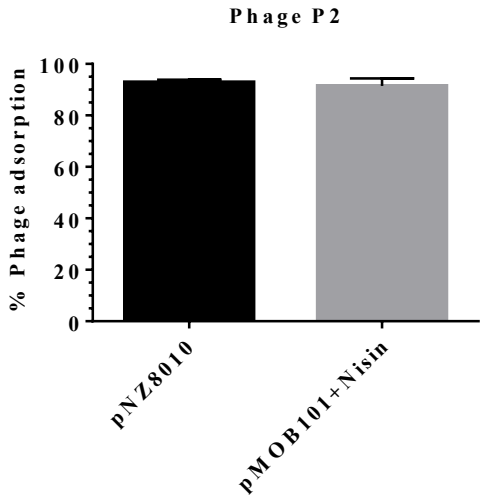


Figure 15. Bacteriophage adsorption assay of p2 in *L. lactis* expressing or not CwpV type II. Bars denote means ± standard deviation (SD) of three independent biological replicates, which were also plated in technical triplicates. Unpaired t-test of data was done with NZ9000 carrying the plasmid pNZ8010 as the reference strain.

3.4 Expression of CwpV in *L. lactis* suggests an increase in cellular aggregation

As previously reported for *C. difficile* strain 630, overexpression of CwpV (all the types apart from type V) induced aggregation in broth at high density (OD_{600} > 10) (Reynolds, Emerson, de la Riva, Fagan, & Fairweather, 2011b). It was showed also that the aggregation occurred even early in the first hour of bacterial growth. The hypothesis for this phenomenon implies that this new reformed bacterial arrangement could come from CwpV-mediated auto-aggregation. Regarding the CwpV expression in *L. lactis* NZ9000, we decided to grow overnight a bacterial culture bearing the plasmid pMOB101 and induced it with 5 ng/mL nisin (de Ruyter et al., 1996). In parallel, we grew another culture but with no induction. The cultures were started with a 1% inoculum, and they were incubated for 24h. The next day we observed that in both cases (with induction or without induction), the expression of *cwpV-II* in *L. lactis* NZ9000 did not produce a macroscopic change regarding aggregation in liquid

suspension. However, in a reproducible manner, we could detect clumping of the bacteria under microscope (for example in Figure 12). More experiments about the CwpV aggregative properties in heterologous host need to be done, as well as its role in bacterial colonization and biofilm formation.

CHAPTER IV

ROLE OF SLPA IN *CLOSTRIDIUM DIFFICILE* BACTERIOPHAGE INFECTION

4. Inactivation of *slpA* in R20291 leads to complete bacteriophage resistance, and its complementation restores bacteriophage susceptibility

With the purpose of understanding the role of the surface protein SlpA in *C. difficile* phage infection, we used a mutant from the epidemic strain R20291 defective for the protein SlpA. This mutant was obtained as a result of a work where genetically modified contractile R-type bacteriocins (called Diffocins) from *C. difficile* were used to kill BI/NAP1/027-type strains. The mutant was isolated because of its resistance to lysis by the Diffocins. As the Diffocins and phages have common structural characteristics, we decided to test the role of *slpA* in phage infection. The mutant has an adenine insertion early in the sequence of the gene that leads to a premature TAA stop codon, creating a truncated protein (unpublished data). In normal infection conditions, the strain R20291 wild type is susceptible to the infection by the *Siphoviridae* family members' ϕCD38-2, ϕCD111, and ϕCD146 (Table 1). Using a spot test assay, we evaluated the result of the absence of SlpA on the susceptibility to infection by these phages. We deposited five microliters of diluted phage lysates (from 10^8 to 10^5 PFU) on top of soft agar overlays containing the R20291 or R202091 *slpA* mutant strains. As a positive control, we used dilutions of Diffocins (Gebhart et al., 2015). We showed that the absence of SlpA from the cell surface rendered the bacterium completely insensitive to infection (Figure 16).

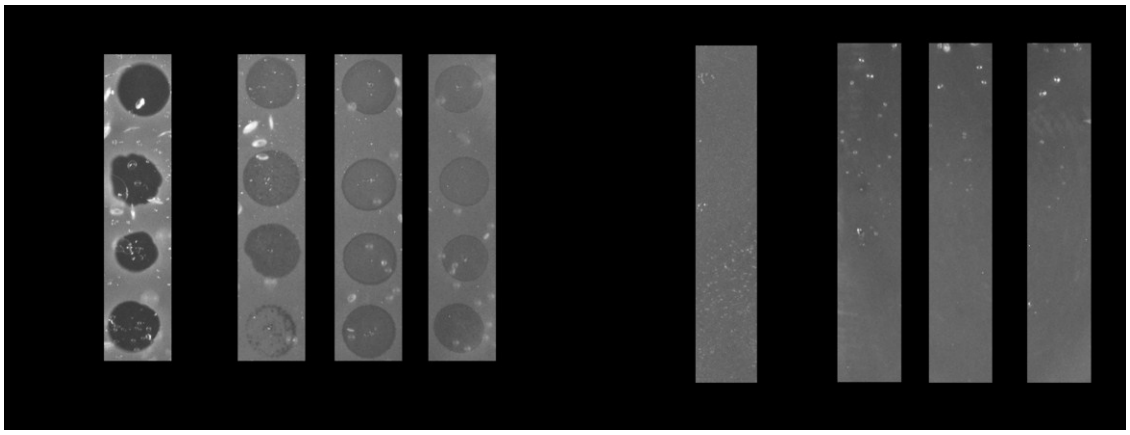


Figure 16. Inactivation of *slpA* in R20291 leads to complete bacteriophage resistance. Bacterial spot tests. Five microliters of diluted phage lysates were deposited on top of soft agar overlays containing log-phase cultures of *C. difficile* strains. The *C. difficile* R20291-specific bacteriocin (Diffocin) was used as a positive control.

However, complementation of the *slpA*$^-$ mutant with a plasmid carrying a wild-type *slpA* allele (CDMOB04Rev) restored susceptibility to phage infection with ϕCD38-2, ϕCD111, and ϕCD146 (Figure 17). In both cases, the *C. difficile* R20291-specific bacteriocin (Diffocin) was used as a positive control (undiluted and dilutions of 1/4, 1/6, 1/10).

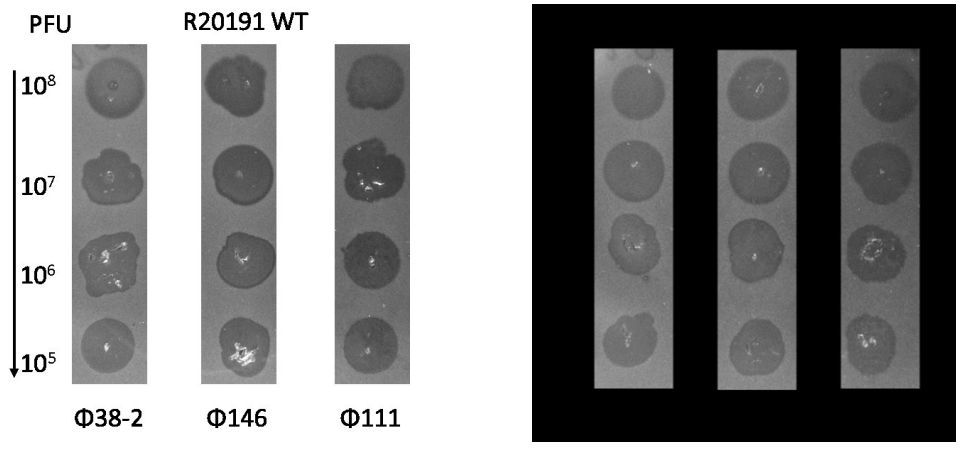


Figure 17. Complemented *slpA* mutant restores bacteriophage sensitivity. Spot test assay on the R20291WT and R20291 *slpA*$^-$ complemented strains.

4.2 The absence of SlpA leads to a reduction in the adsorption of bacteriophages ϕCD38-2 and ϕCD146, but not ϕCD111

As stated earlier, phage adsorption is the first step of the viral infection cycle and one of the most important. This step is multifactorial and involves complex mechanisms that lead to the entry of the foreign genetic material entering to the cytoplasm. Considering the SlpA localization and the fact that this protein is an integral part of the bacterial cell-wall, we hypothesized that its absence could affect the phage attachment to the bacterial surface. We thus performed a phage adsorption assay with the R20291 and the R20291 *slpA*$^{-}$ strains. In Figure 18 we demonstrated with a phage adsorption assay that the absence of the protein SlpA in the *slpA*$^{-}$ mutant led to a significant reduction of about 30 to 40% in the adsorption of ϕCD38-2 (70.6 ± 8.1% compared to 96.4 ± 2.1% for the WT) and ϕCD146 (56.1 ± 18.6% compared to 90,8 ± 6,1% for the WT), respectively, whereas adsorption of ϕCD111 was not significantly affected (94.8 ± 3.1 % compared to 97.7 ± 1.1 % for the WT). This result suggests that although the absence of SlpA from the bacterial cell wall reduces the attachment of ϕCD38-2 and ϕCD146, the level of adsorbed phage is still important, suggesting that SlpA does not dictate alone the fate of the infection process. For the phage ϕCD111, there was no significant reduction, suggesting a slightly different mechanism of host recognition, despite the requirement for SlpA.

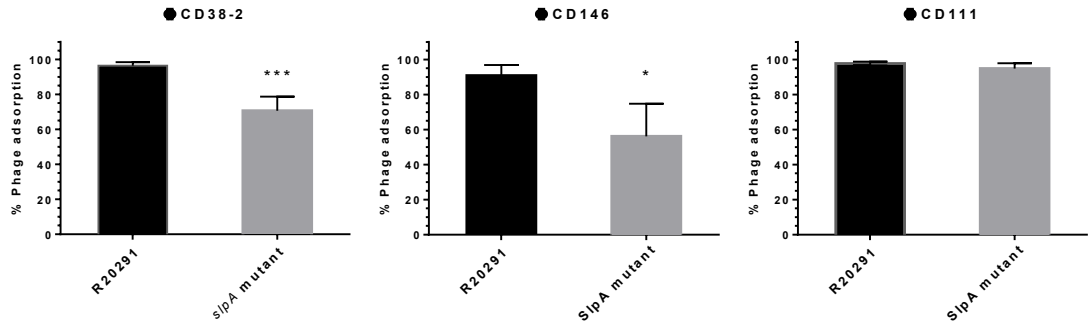


Figure 18. The absence of SlpA leads to a reduction in the bacteriophage adsorption of ϕCD38-2 and ϕCD146, but not ϕCD111. Adsorption assays. Bacteria from an overnight culture were inoculated in TY broth and grown until exponential phase. Free bacteriophages in the supernatant that did not adsorb were counted on standard soft agar overlays and titers were compared with the initial bacteriophage inoculum. Bars denote means ± standard deviation (SD) of three independent biological replicates, which were also plated in technical triplicates. Unpaired t-test of data was done with R20291 WT as the reference strain. (*P < 0.05).

4.3 Complementation of the R20291 *slpA*$^-$ mutant with different *slpA* alleles changes the susceptibility to bacteriophage infection

The cell surface is composed of a paracrystalline proteinaceous S-layer encoded by the *slpA* gene within the *cwp* gene cluster. The diversity and evolution of *slpA* and adjacent genes that also encode immunodominant cell surface antigens have been well characterized (Kate E Dingle et al., 2013; Tuomo Karjalainen et al., 2002). We sought to expand our knowledge in the host range effect of the various SlpA types in the infection by phages from two families (*Siphoviridae* and *Myoviridae*), using the advantage of having the same genetic background of strain R20291, which is only infected by *Siphoviridae* viruses but not *Myoviridae* viruses (this pattern possibly emerging due to the presence of antiphage systems or because the *slpA* type is not the appropriate one for these phages). We transferred by conjugation each of the 12 types of *slpA* alleles into the strain R20291 *slpA*$^-$ mutant and assessed infection by phages ϕCD38-2, ϕCD111, ϕCD146, ϕCD481-1, ϕCD481-2, ϕCD505, ϕCD506, ϕCD508, ϕMMP01, ϕMMP02, ϕMMP03, ϕMMP04, and ϕCD52, using spot tests. The selection of these particular phages was based on the expected CRISPRs interference of the strain R20291

against the viruses of our phage bank, derived from our previous study (Boudry et al., 2015). Table 5 summarizes the results observed for each combination of *slpA* type/phage.

Table 5. Complementation of the R20291 *slpA*$^-$ mutant with different *slpA* alleles changes the susceptibility to bacteriophage infection.

Strain name	*slpA* cassette	ΦCD38-2	ΦCD111	ΦCD146	ΦCD481-1	ΦCD481-2	ΦCD505	ΦCD506	ΦCD508	ΦMMP01	ΦMMP02	ΦMMP03	ΦMMP04	ΦCD52
R20291 *slpA*$^-$	No SlpA	-	-	-	-	-	-	-	-	-	-	-	-	-
R20291	4 (wild type)	++	+++	+++	-	-	-	-	-	-	-	-	-	-
CDMOB01	1	-	-	-	-	-	-	+	+	-	-	-	+	-
CDMOB02	2	-	-	-	-	-	-	-	-	-	-	-	-	-
CDMOB06	6	-	-	-	-	-	-	+	+	-	-	-	-	-
CDMOB07	7	-	-	-	-	-	-	-	+	-	-	-	-	-
CDMOB07b	7b	-	-	-	-	-	-	-	-	-	-	-	-	-
CDMOB08	8	++	+++	+++	-	-	-	-	-	-	-	-	-	-
CDMOB09	9	++	+++	+++	-	-	-	-	-	-	-	-	-	-
CDMOB10	10	-	-	-	-	-	-	+++	+	-	-	-	-	-
CDMOB11	11	++	+++	+++	-	-	-	-	-	-	-	-	-	-
CDMOB12	12	-	-	-	-	-	-	-	+++	-	-	-	-	-
CDMOB13	13	++	+++	+++	-	-	-	-	-	-	-	-	-	-
CDMOBH2/6	H2/6	-	-	-	-	-	-	-	+	-	+	+	-	-
	Phage family	S	S	S	M	M	M	M	M	M	M	M	M	M

Host range analysis. Isolate sensitivity (indicated as follows: +, sensitive; ++, moderately sensitive; +++, very sensitive; -, not sensitive.) is based on the intensity of the clearing zones. Bacteriophage families: M, *Myoviridae*; S, *Siphoviridae*.

Firstly, the host range matrix shows that the R20291 *slpA*$^-$ mutant was resistant to all phages tested, independent from their family, i.e. *Siphoviridae* or *Myoviridae*, suggesting that SlpA is a common phage receptor used by most, if not all phages infecting *C. difficile* (although there are other factors that determine the natural host range). Second, as previously emphasized, the complementation with the type 4 *slpA* allele (same as the wild-type strain R20291) restored the susceptibility to phages ϕCD38-2, ϕCD111, and ϕCD146. Similarly,

the previous resistance to all phages of the *Myoviridae* family was maintained. Interestingly, complementation with the *slpA* alleles 8, 9, 11 and 13 restored the host range in an identical manner as the allele 4 did, suggesting that the three siphophages tested can use more than one *slpA* allele as a receptor to infect different hosts.

Complementation with allele types 1, 6 and 10 made the R20291 strain susceptible to infection by the myophage ϕCD506, which has never been observed before. Similar observations were also made with the allele types 1, 6, 7, 10, 12 and H2/6 that conferred susceptibility to infection by phage ϕCD508. The allele type H2/6 allowed infection by phages ϕMMP02 and ϕMMP03, and allele type 1 allowed phage ϕMMP04 to infect the R20291 complemented strain.

Of note, the *slpA* types 10 and 12 gave a strong bacterial lysis with phage ϕCD506 and ϕCD508, respectively, indicating that the complementation with those *slpA* types rendered the bacteria very sensitive to these phages. In all cases, there was no evidence in previous investigations of phages of the *Myoviridae* family infecting the epidemic strains R20291, besides the endogenous phi027 prophage, which is predicted to be a member of this family. These results thus indicate that SlpA is a primary determinant for phage infection and that the *slpA* type expressed in the cell-wall determines the susceptibility of bacterial cells to infection by different viruses.

4.4 SlpA co-expression and host range prediction.

Understanding that the SlpA protein expression on the bacterial surface of *C. difficile* could define its sensitivity to known phages, as well as phages from different families, we decided to test if a co-expression of two different *slpA* alleles would give to the bacteria a dual host-range sensitivity. For this, we utilized the wild type strain R20291 (already expressing its natural chromosomal *slpA* type 4), and we conjugated it with the plasmid pRPF185 bearing the *slpA* allele type 12. Using a spot test assay, we evaluated the double susceptibility and obtained as a result that the co-expression of *slpA* type 4 and type 12 conferred susceptibility to phages ϕCD38-2, ϕCD111, and ϕCD146 (from the *Siphoviridae* family) in addition to the phage ϕCD508 (from the *Myoviridae* family) (Figure 19). As far as we know, this is the first

time that manipulation of the expression of a cell-wall protein not only brings about a new host range to a specific strain but also that co-expression of two different *slpA* alleles can lead to a combined susceptibility phenotype to a single strain.

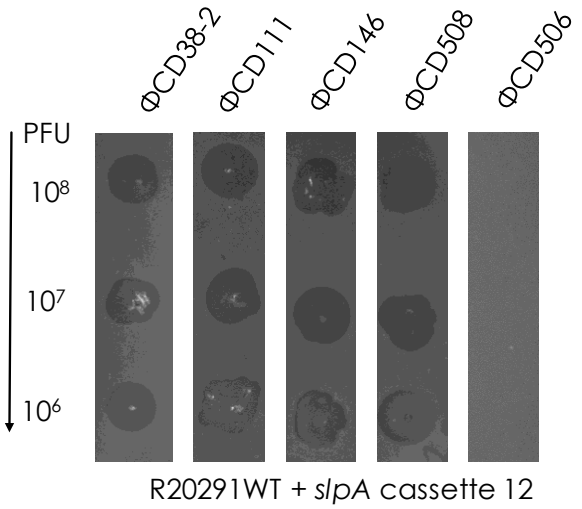


Figure 19. Co-expression of *slpA* type 12 in R20291 confers double susceptibility. Spot test assay on the R20291WT complemented with the slpA cassette 12.

CHAPTER V

DISCUSSION AND CONCLUSION

5. Discussion

Microorganism evolution depends on a complex multitude of factors including the interactions with other microorganisms and genetic elements (like viruses and plasmids). In host-parasite relationships, each microorganism causes a reciprocal selective pressure on each other. The result of this dynamic is an endless arsenal of biological mechanisms that could ensure survival or in Lewis Carroll's words "*it takes all the running you can do to keep in the same place*". The main idea of this work was comprehending and delving into the infection mechanisms of the phages of the Gram-positive bacterium, *C. difficile*. More specifically, we wanted to test the activity of the antiphage protein CwpV from *C. difficile* into the heterologous host *L. lactis*. Indeed, this bacterial species is susceptible to several strictly lytic phages, and genetic manipulations are easier. In addition, we wanted to confirm a long-standing question as to whether phages could use the SlpA protein as a receptor to initiate infection.

It is relevant to understand the effect of phages in the biology and ecology of bacterial strains that have importance to human health, such as *C. difficile*. Using *L. lactis* (an important bacterial species in the dairy industry), we provided experimental evidence in which we verified that the cell-wall protein CwpV from *C. difficile* protected *L. lactis* from phage infection. In parallel, we sought to understand the role of SlpA as a possible receptor, in phage infection and how the different *slpA* alleles could modify susceptibility to phage infection.

We showed that the expression of CwpV type II protects against one lactococcal phage of the 936 group. Further data shows that the phage is capable of adsorbing to the bacterial host, suggesting that the inhibition of the replication cycle happens after the adsorption: at the phage DNA injection or another downstream process. Like it was already stated in previous

work from our laboratory (Sekulovic et al., 2015a), the action of CwpV is similar to other phage-derived resistance systems (Sie systems) (S. J. Labrie et al., 2010; Mahony, McGrath, Fitzgerald, & van Sinderen, 2008; McGrath, Fitzgerald, & van Sinderen, 2002). Nisin regulated and constitutive expression of CwpV-II from two different plasmids was enough to block the infection by phage p2. However, expression from the constitutive vector pRPF144E showed a reduced protection in spot test assays. Possibly this difference between protein expression was due to the non-optimal maintenance of pRPF vectors in *Lactococcus* hosts. The proper expression of CwpV is necessary for complete protection, and perhaps the phage-to-host ratio is fundamental for optimal protection.

As we previously hypothesized in other work with CwpV, it is possible that for the antiphage action from CwpV, the protein should cover a large proportion of the cell surface. The natural expression of CwpV in wild type strains account for only 13.3% of the total cell-wall proteins (where the rest is mainly accounted by cell wall protein SlpA that forms a two-dimensional paracrystalline array in which CwpV is embedded) (Reynolds et al., 2011b). Consequently, a suboptimal expression of the protein from a pRFP plasmid could lead to zones where the bacterial cell surface may not display CwpV, and that would explain why at high MOI, phages can manage to infect the cell. There are still fundamental questions to address regarding the MOI in controlled infections and the strength of antiphage systems (here the latter factor tends to be taken more into account).

In addition, the dose-dependent response observed in the protein induction for the plasmid pNZ8010 has been determined in other phage systems from lactic acid bacteria. Where transferring the "antiphage" gene into a well establish multi-copy plasmid leads to a stronger phage resistance phenotype (McGrath et al., 2002; Sun, Göhler, Heller, & Neve, 2006a). For example, the Sie2009 system was shown to cause complete resistance against p2 when expressed from the nisin-inducible expression vector pNZ8048 (Mahony *et al*, 2008).

Then we showed that when CwpV is expressed in *L. lactis*, it conserved in a certain measure its antiphage activity, in this case toward the phage p2. The obtained result did not reach EOP values from previous studies with *C. difficile* siphophages where values $< 5 \times 10^{-7}$ for the

phage φCD38-2 were obtained (Sekulovic et al., 2015b). Nevertheless, the EOP values were similar to phages of the *Myoviridae* family where CwpV (types I, III, V) provided some protection against the phage φMMP01 (with EOPs of 9 x 10^{-3}, 5.6 x 10^{-2}, 9.7 x 10^{-2} respectively). (Sekulovic et al., 2015a). Although the phage p2 is a siphophage, its genetics and ecology vastly differs from *C. difficile* phages. It is especially remarkable that CwpV is active against phages that can infect such divergent hosts, this could indicate that CwpV interferes with a conserved phage structure, host element, or step in the infection process common in both groups of phages.

It is not the first time that an antiphage system is transferred to a heterologous host. The Ltp protein that is encoded by the *Streptococcus thermophilus* phage TP-J34 is a Sie system (EOP of about 10^{-2} in *S. thermophilus*) that once transferred to *L. lactis*, can render the bacteria resistant to the phage P008 (from the 936 group) (EOP < 10^{-9} in *L. lactis*), but not to other members of lactococcal phage species (Mahony et al., 2008; Sun, Göhler, Heller, & Neve, 2006b). Ltp is a 42 amino acids lipoprotein-associated to the cell membrane that presents similarities to Sie2009 (already discussed in the section 1.9.4.3)

The mechanism of infection of phage p2 starts when viral particles possibly dock to the cell-wall: firstly, having a weak interaction between the tail adhesins and the bacterial pellicle (an outer layer composed of cell-wall polysaccharides). Later the specific interaction of the phage receptor binding protein (RBP) in the baseplate and the pellicle occurs. Leading finally to the baseplate activation, RBP molecular rotation and strong attaching to the saccharide receptor (Cecilia Bebeacua et al., 2013). Therefore, regarding the mechanism in which Sie systems operate, one of our first hypothesis was that CwpV could be masking an important feature of the cell-wall that activates the DNA injection or possibly blocks the adsorption of the viral particle to the host, therefore preventing the RBP from interacting with the sugar receptor of phage p2.

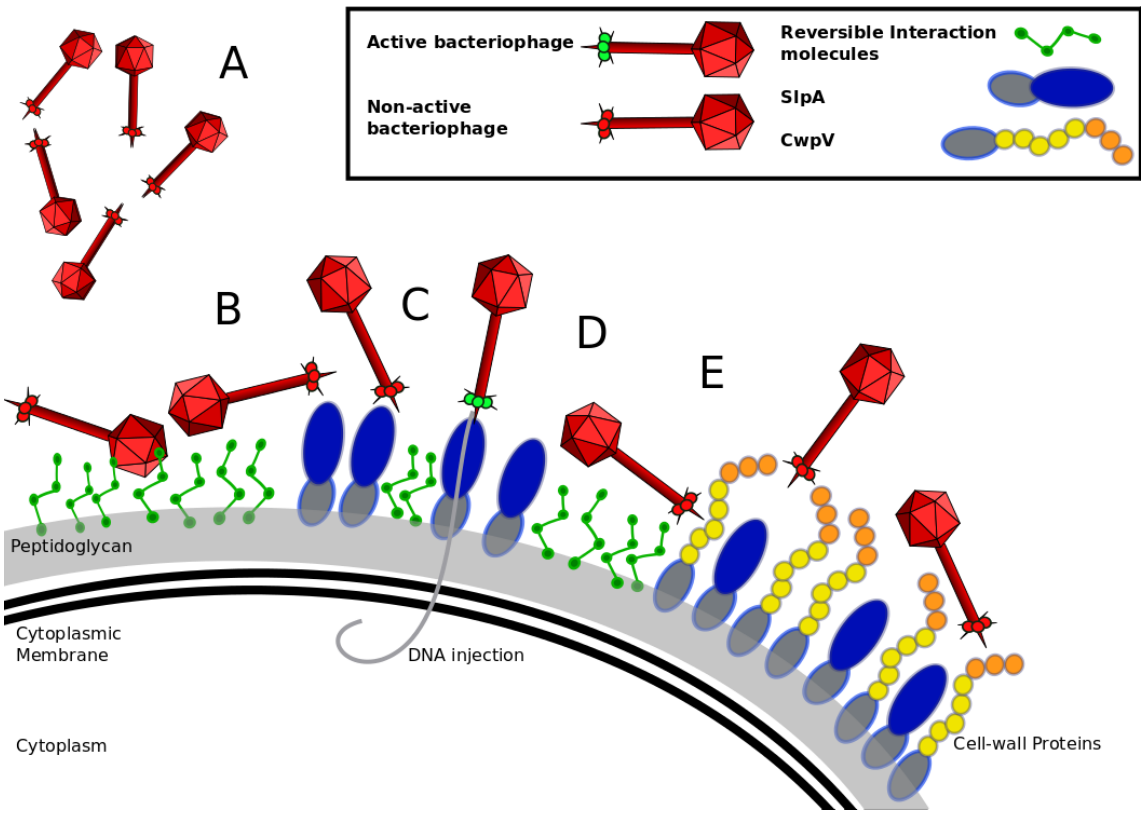


Figure 20. Proposed generalist mechanism of infection by a bacteriophage under a bacterial host expressing SlpA or CwpV proteins. (A) Free bacteriophage roam the environment for a suitable host. (B) Reversible interaction among the host cell wall (unknown) molecules and bacteriophage structural proteins is established (tail or capsid). (C) Specific interactions with SlpA as a secondary receptor may lead the final bacteriophage positioning. (D) Correct interplay between the bacteriophage RBP and the SlpA molecule guide the secondary binding and structural activation that lead to the injection of the viral DNA. Absence of the SlpA renders the host insensitive to the bacteriophage but the viral particle can still attach to the cell up to a certain extent. (D) (E) In the context of CwpV expression, the presence of the protein in the cell-wall impedes bacteriophage infection. This phenomenon may happen by preventing the bacteriophage from reaching the SlpA receptor or on the other hand by the direct interaction of CwpV repetitions with a structural component of the viral particle.

The data obtained in this work suggest that CwpV possibly interferes at the moment of the DNA injection (Figure 20). Our experiments demonstrated that adsorption of the phage was not prevented in *L. lactis* expressing CwpV, suggesting that CwpV does not impede the first steps of infection (unspecific, specific interactions and receptor binding). Previous research in *C. difficile* did not detect phage replication in cells expressing CwpV (Sekulovic et al.,

2015a), hence the most logical possibility points toward an interference of CwpV on the phage DNA injection. We hypothesize that this specific phenomenon could happen when CwpV interacts with a structural element of the phage tail (tail fibers, tail tube, baseplate), which could be structurally conserved among phage families (at least with the phages used in this and previous studies).

Our heterologous host gives us an outstanding tool to unveil more details in the future of our theoretical infection model. Contrary to *C. difficile* phages, the knowledge on the biology and structure of *L. lactis* phages is very well studied. The bacterial receptor of p2 and related phages is already discovered. Phage p2 infects *L. lactis* using a RBP located in the distal part of its proteinic tail. The RBP is composed of homotrimeric proteins which form three domains (Spinelli et al., 2006). One of the three domains is called the receptor-recognition head (a seven-stranded beta-barrel). This domain has a high affinity for a specific pellicle phospho- polysaccharide of *L. lactis*, which acts as a bacterial receptor (Tremblay et al., 2006). These features make the heterologous host *L. lactis* and phage p2 an interesting model to investigate superinfection exclusion (Sie) systems.

The previous data do highlight not only the potential use of this model as a heterologous host (and antiphage testing) but also shows the broad spectrum that some Sie systems have. Mutants of the lactococcal phage P008 capable of circumventing the streptococcal Ltp antiphage activity were already isolated. Genome sequencing showed specific mutations in the gene encoding the tape measure protein (TMP), suggesting that Ltp targets this particular protein. Still, there is no direct experimental evidence of such interaction (C. Bebeacua et al., 2013). Although it is tantalizing to suggest a possible interaction between CwpV and the TMP, additional experiments will be required to demonstrate such interaction. Our previous data in *C. difficile* agree with this, a partially truncated type of CwpV in which five of the C-terminal repeats have been removed has an attenuated antiphage activity against siphophages, while on the contrary, a complete depletion of the C-terminal repeats causes a total loss of activity (Sekulovic et al., 2015a).

To date, it is still essential to isolate phages capable of overcoming the antiphage activity of CwpV in both *C. difficile* and *L. lactis* models. Mutant isolation can give us clues about the possible phage targets and determine the specific interactions of CwpV and phage components. Up to now, we did not isolate such mutants when phage protection was maximal, but additional work would be required. Equally, there are a plethora of well-known lactococcal phages that can be tested (M13, 712, C2 SK1, Eb1), as well as other CwpV types (I, III, IV, V). These assays are underway.

Care and prevention plans for infection diseases rely massively on conventional antibiotic therapy, which inflicts an adamant selection for bacterial resistance and interfere with the "normal" state of protective microbiota. Consequently, there has been an emergence of opportunistic pathogens, such as *C. difficile*. These bacteria can exploit antibiotic-derived gut microbiota disturbances to grow and cause disease. In a particular study (Gebhart et al., 2015), the researchers have developed alternative macromolecular agents that use contractile bactericidal protein complexes also known as phage-like particles (R-type bacteriocins) to neutralize particular *C. difficile* pathogens. Efficacy in a preclinical animal study point out that these phage-like particles secure further improvement as possible prophylactic molecules to impede *C. difficile* diseases in humans. Further investigation on the same subject (data not published) shows that the receptor utilized by those phage-like particles is the SlpA protein and the specific type of SlpA determines the lysis capabilities of a particular phage-like particle. Taking those results together and understanding that phages and phage-like particles share numerous structural features, the aim of our study was to establish the role of the S-layer protein SlpA in phage infection of *C. difficile*. We obtained the first evidence of SlpA serving as a receptor for infection by *Siphoviridae* phages (φCD38-2, φCD111, and φCD146). Further data shows that SlpA also functions as a receptor for *Myoviridae* phages (phages ϕCD506 and ϕCD508).

Until now, bacterial receptor(s) recognized by these phages to infect *C. difficile* were unknown. We hypothesized that such receptor was a surface carbohydrate, a membrane protein, or another surface constituent. Using a *slpA*$^{-}$ mutant from the epidemic strain R20291, we show that the absence of SlpA protein from the cell-wall surface renders the bacterial

population completely insensitive to infection by three related phages of the *Siphoviridae* family: ϕCD38-2, ϕCD111, and ϕCD146. This phage resistance was observed in spot-test and broth experiments. With the objective of recovering the infection phenotype against the same phages, we performed a complementation of the *slpA*$^{-}$ mutant with a wild-type *slpA* allele (type 4) that ultimately restored the previous susceptibility to phage infection (model of infection in Figure 21A, B, C).

Subsequently, our experiments demonstrated a significant reduction of about 30 to 50% in the adsorption of ϕCD38-2 and ϕCD146 phages respectively, whereas adsorption of ϕCD111 was unaffected. The interplay of the blockage of ϕCD111 and its adsorption are still not understood. Equally, for ϕCD38-2 and ϕCD146 it is still possible that a diminution, but not a complete depletion in the adsorption level of a phage to a host could affect significantly enough the infection process to prevent a detectable infection. This kind of phenomenon has been registered already in *L. lactis*., where the phage bIL67 from the genus *C2virus* does not infect very well the strain DGCC7271 (EOP $<1\times10^{-8}$), however it attaches to the cell reaching an adsorption rate of 67.3±8.0 % (Millen & Romero, 2016). Interestingly, although *C. difficile* siphophages are closely related in structure and gene homology, they do not share the same host spectra (Sekulovic et al., 2014). Our data suggest that slight genetic differences (point mutations, small indels) account for the different adsorption values of the phage ϕCD111 respecting ϕCD38-2 and ϕCD146.

Phages are omnipresent in the gut ecosystem. Important efforts in the last decade have aimed to understand the role of viral populations in the control and shape of local resident bacterial populations (microbiota), that are ultimately linked to the heath state of an individual. From that perspective, investigating the "port of entry" of these phages to the bacterial cell is of extreme importance. Equally significant, thanks to PCR technics and genome sequencing, the epidemiology, evolution, and genetic diversity of *C. difficile* are better understood. *C. difficile* has a diverse population structure that results in hundreds of strain types. Researchers using whole-genome sequencing (Kate E Dingle et al., 2013) of 57 *C. difficile* isolates, representative of the population structure and various clinical phenotypes, performed phylogenetic analyses on the genomic regions (>63 kb) including the *cwp* cluster (and *slpA*

inside). The research stated a high genetic diversity across the *cwp* cluster peaked within the *slpA* gene, *cwp66* (adhesin), and *secA2* (secretory translocase). These genes formed a cassette, and 12 different variants were recognized. We transformed and complemented by conjugation the R20291 *slpA*$^{-}$ mutant with each of the 12 *slpA* alleles. We determined that complementation of the *slpA*$^{-}$ mutant with 4 (out of 12) variant slpA alleles restored susceptibility to ϕCD146, ϕCD38-2, and ϕCD111 phage infection. Nevertheless, even more interesting, five of the twelve alleles conferred susceptibility to other phages of the *Myoviridae* family that have never been reported to infect the R20291 strain. Understanding the last results, we wanted to predict if the co-expression of two different types of SlpA could render a population of bacteria multi-sensitive to two groups of phages. We observed that the co-expression of the slpA type 12 and type 4 (naturally expressed from the chromosomal gene) in R20291 confers double susceptibility to phages of two different viral families. To our knowledge, this is the first time the expression of SlpA in a particular *C. difficile* strain can render a strain sensitive to structurally and genetically different phage groups. As of today, hundreds of strains have been characterized and sequenced. Yet, many strains are still to discover and their characterization might lead to the identification of new configurations of *slpA* and *cwpV* alleles. On the other side of the equation, *C. difficile* phage characterization is still a field in development. Taking all this together, it is necessary to describe all these bacterial strains and phages. The more information we gather about them, the more understanding we will get regarding the role of the bacterial surface proteins in phage infection. Finally, phage-host interaction is a highly complex field. Susceptibility to a particular phage does not rely solely on the presence of a suitable surface receptor. Also, the presence of CRISPR systems, R-M systems, and other prophages that can provide repressor-mediated immunity, defines the host spectrum of a strain.

5.2 Conclusion

In natural conditions and in an ecological context, bacteria and phages are constantly evolving (dying or becoming resistant). Phage infection creates an evolutionary pressure on bacteria to innovate different means of survival. Adsorption impairment, blocking DNA injection, R-M systems, CRISPR-Cas systems among others are the result of the infinite evolutionary war of adaptation and counter-adaptation. From these mechanisms, regular and

phase-variable antiphage (Sie) systems such as CwpV emerges as highly relevant in circumstances where multiple phage predators are present in the environment. The development and establishment of study models is critical to understand such evolutionary relationships and resultant mechanisms. Having that in mind, *L. lactis* is a crucial tool to test other lactococcal phages along with other types of CwpV to determine the specific interactions among this anti-phage system and bacterial viruses. Although promising candidates for phage protection have been in development in recent years (for example the well-known Abi systems), this anti-phage system can be transferred to important industrial hosts (as *L. lactis* is in the dairy industry) to be used in culture protection and other biotechnological applications where phage attacks cause economic burden. On the other hand, clinical and *in vivo* tests are necessary to understand how a phase-variable antiphage system can protect subpopulations of bacteria from lytic phage attacks during gut colonization. In the same order of ideas, it is of fundamental importance to understand how phages enter to the cell and which receptors they use to complete the viral replication cycle.

The fast emergence of highly resistant bacterial pathogens is occurring word-wide due to the overuse or misuse of antibiotics. This crisis imposes a threat that could kill millions of persons. New drug development by the pharmaceutical industry does not catch up with the apparition of emergent resistant bacteria. In this way, phage therapy (the therapeutic use of lytic phages to treat bacterial infections) represents an interesting alternative to antibiotics. Phages can be targeted more specifically than most antibiotics, thus not affecting other important bacteria and the imbalance of the gut microbiota. However, in the context of this research, this knowledge could lead to a panorama where the usage of phages can be ameliorated. Firstly, by sequencing strains and looking for specific types of *slpA* and selecting phages that can use that *slpA* to enter to cell and lyse the host. Second, and most importantly, by phage RBP swapping. In absence of appropriate natural phages, changing the host range of a phage by swapping the RBP from other phages could become a useful tool for delivering genetically modified phages (in phage therapy cocktails) that could be directed to *C. difficile* during gut infection.

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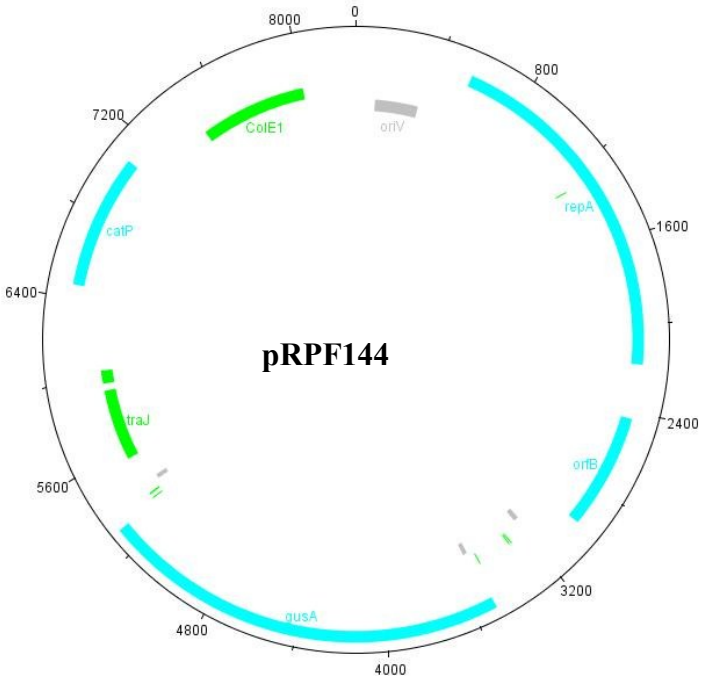

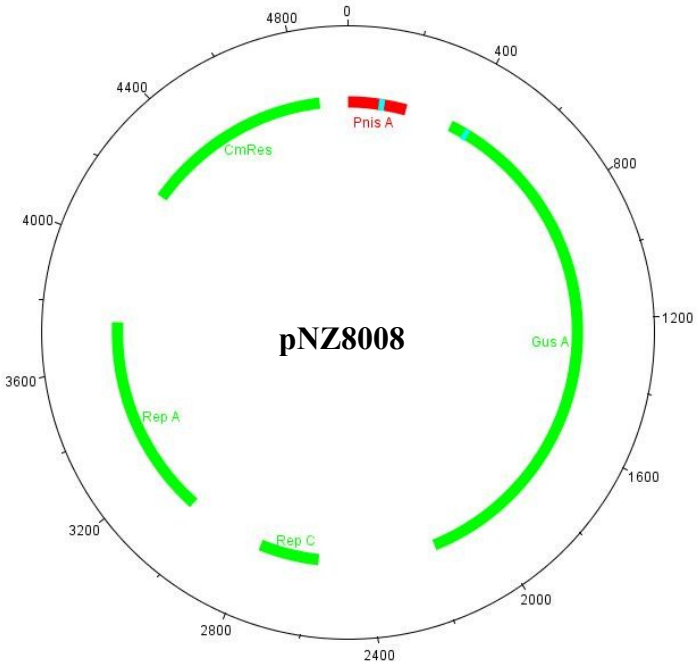

Supplementary figure 1. pRPF144E is a pRPF144 plasmid without *gusA* and with a unique BamHI site, also pNZ8010 is a pNZ8008 plasmid without *gusA* and with a unique MCS1.